Differentiation of *Neisseria gonorrhoeae* strains by polymerase chain reaction and restriction fragment length polymorphism of outer membrane protein IB genes

Q C Lau, V T K Chow, C L Poh

Abstract

***Objectives*—To employ polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis for the rapid differentiation of *Neisseria gonorrhoeae* protein IB (PIB) isolates and to compare its usefulness with the widely accepted auxotype/serovar classification scheme.**

***Methods*—The outer membrane protein IB genes of 47 gonococcal isolates belonging to 10 different serovars were amplified by PCR. The ~1 kb DNA products were then digested separately with restriction enzymes *Cfo*I and *MspA1*I, and electrophoresed on agarose gels.**

***Results*—Cleavage of PIB genes by *MspA1*I and *Cfo*I differentiated all the *N gonorrhoeae* strains into five and six PCR-RFLP profiles, respectively. PCR-RFLP was more discriminatory than auxotyping, which classifies the strains into only two auxotypes. Some strains belonging to common serovars could be further differentiated. A combination of PCR-RFLP analysis, auxotyping and serotyping further increased the discrimination of the strains into 34 subtypes. The PCR-RFLP method was easy to perform, reliable, reproducible, and consistent with published nucleotide sequence data.**

***Conclusion*—The PCR-RFLP method can augment auxotyping and serotyping or be used as a preliminary screening tool to differentiate *N gonorrhoeae* strains in areas where serotyping reagents are not easily available.**

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Keywords: *Neisseria gonorrhoeae*, outer membrane protein IB, PCR, RFLP, serotyping, auxotyping

Department of Microbiology, Faculty of Medicine, National University of Singapore, Lower Kent Ridge Road, Singapore 0511, Republic of Singapore
Q C Lau
V T K Chow
C L Poh

Address correspondence to: Dr V T K Chow, Department of Microbiology, Faculty of Medicine, National University of Singapore, Lower Kent Ridge Road, Singapore 0511, Republic of Singapore

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Introduction

Neisseria gonorrhoeae is the causative agent of gonorrhoea, a common sexually transmitted disease worldwide. Preventing the spread of this infectious disease is dependent upon antimicrobial treatment of infected persons, better education of the general public and effective epidemiological tools for monitoring gonococcal strains. Various typing schemes have been developed for the characterisation of *N gonorrhoeae* isolates. Currently, the most widely accepted classification scheme is based on the dual combination of auxotyping and serotyping.[1] Auxotyping has its limitations as most Asian isolates are either proline-requiring (Pro^-) or prototrophic.[2] Serotyping is based on the differential recognition of separate panels of monoclonal antibodies (mAbs) against the outer membrane proteins IA (PIA) or IB (PIB) of *N gonorrhoeae* strains. Serotyping is occasionally subjective and may be affected by batch-to-batch variation of mAbs.[3] Moreover, predominant PIB serovars such as IB-3 and IB-7 may not be sufficiently discriminated, while other serovars may be overdiscriminated.[4] Recently, polymerase chain reaction (PCR) has gained popularity for the characterisation of strains belonging to certain bacterial species. Random amplification of polymorphic DNA (RAPD), also known as arbitrarily-primed PCR (AP-PCR) has been used to differentiate strains of *Helicobacter pylori*[5], *Listeria monocytogenes*[6] and *Clostridium difficile*[7]. RAPD is a rapid and simple technique but is often fraught with the generation of artifactual (false positive, false negative, or both) bands which can bias data interpretation.[8]

We describe here a method based on PCR amplification of the gonococcal outer membrane PIB gene combined with restriction fragment length polymorphism (RFLP) analysis that can further differentiate strains belonging to the major PIB serovars of *N gonorrhoeae*.

Materials and methods

Gonococcal strains Forty seven random isolates obtained from male and female patients attending a local STD clinic between June and August 1986 were selected for this study. The culture conditions and DNA extraction have been described previously.[4,9]

Auxotyping and serotyping The strains were auxotyped, and serotyped with a panel of six mAbs specific for PIB[1] (tables 1 and 2). There were only two auxotypes: 17 strains were prototrophic (S4, S7, S12, S21, S22, S25, S31, S33, S36, S39, S48, S50, S52, S53, S56, S57 and S58) and 30 strains were proline-requiring (S1, S2, S3, S5, S8, S10, S11, S16, S17, S18, S19, S20, S24, S26, S27, S28, S30, S32,

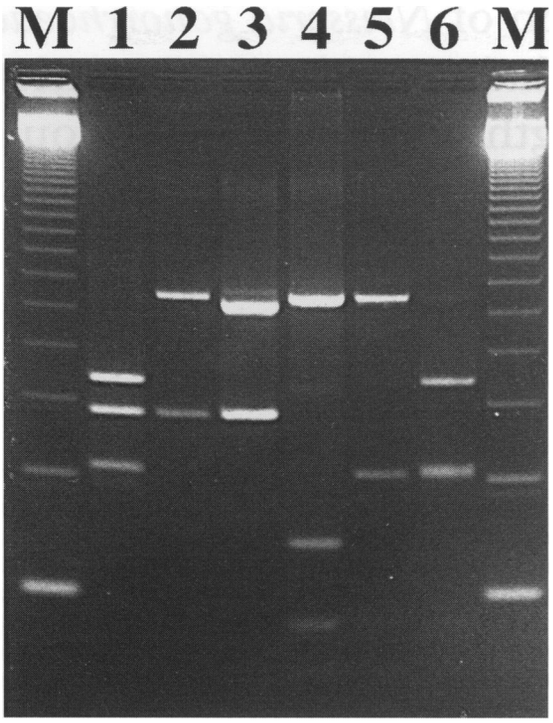

Figure 1 Agarose gel electrophoresis of PIB gene PCR products cleaved with CfoI. Representative CfoI PCR-RFLP profiles I-VI (table 1) are shown in lanes 1–6, respectively. Lanes M: 123 bp DNA ladder marker.

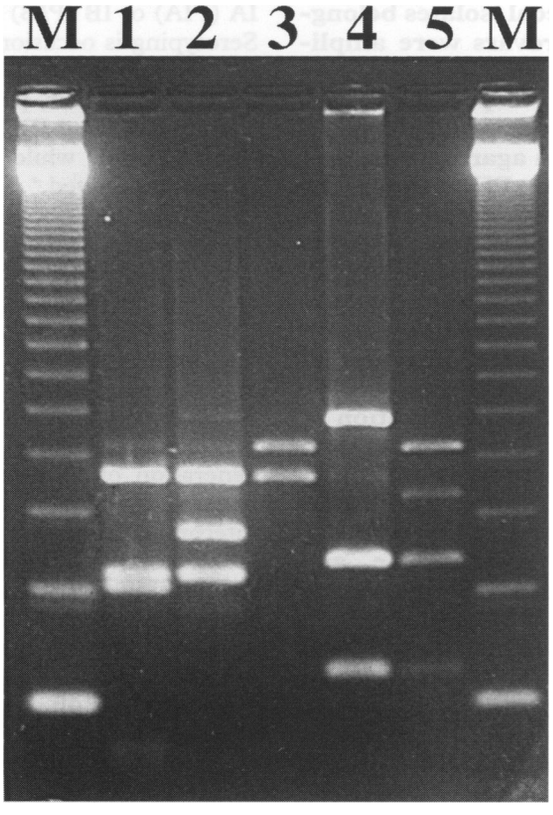

Figure 2 Agarose gel electrophoresis of PIB gene PCR products cleaved with MspA1I. Representative MspA1I PCR-RFLP profiles I-V (table 2) are shown in lanes 1–5, respectively. Lanes M: 123 bp DNA ladder marker.

S34, S35, S37, S38, S40, S41, S43, S44, S45, S46, S51 and S55).

Oligonucleotide primers A pair of PCR primers OMG3 (5′ TAACCAAAAAAGGAATAC-AGCA 3′) and OMG8 (5′ CAGGCTTT TTGTTGATACCA 3′), corresponding to nucleotides 49–70 and 1148–1129[10], respectively was employed to amplify ~1 kb of the PIB gene of *N gonorrhoeae*. The primers were chemically synthesised and HPLC-purified (New England Biolabs, Beverly, MA, USA).

PCR amplification PCR was performed in a 50 μl reaction mixture containing 1 × SuperTaq buffer, 0·25 U of SuperTaq DNA polymerase (HT Biotechnology, Cambridge, UK), 0·2 μM of each primer, 0·2 mM each of the four dNTPs and 50 ng of bacterial DNA, subjected to 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 1 min. Prior to restriction enzyme digestion, 5 μl of each amplified product were electrophoresed on 2% agarose gels stained with ethidium bromide.

Selection of restriction endonucleases Published nucleotide sequences of PIB genes of 10 *N gonorrhoeae* strains S7, S386, S11, S34, S22, S16, S36, MS11, P9 and R10 (belonging to serovars IB-1, IB-2, IB-4, IB-5, IB-7, IB-14, IB-19, IB-9, IB-26 and IB-3, respectively)[10–13] were analysed using the DNASIS software programme (Hitachi, Brisbane, CA, USA) to search for useful restriction enzyme cleavage sites. The enzymes *Cfo*I and *MspA1*I (whose recognition sequences are GCG↓C and C(A/C)G↓C(G/T)G, respectively) were thus chosen on the basis of their ability to generate suitable restriction profiles of the target fragments. Other enzymes such as *Sau3A*I, *Hpa*II and *Kpn*I have been used by Gill *et al*[14] to differentiate between PIA, PIB and PIA/PIB hybrid strains. However, they were unsuitable for differentiating PIB strains as *Sau3A*I does not have cleavage sites in any of the 10 published strains, while *Kpn*I has only one site, and *Hpa*II has between 9–11 sites which generate too many restriction fragments of similar sizes.

Restriction enzyme digestion To the remaining volume of PCR product, 1 × restriction enzyme buffer and 10 U of the corresponding restriction enzyme were added, and incubated at 37°C for 5 hours. The digested products were electrophoresed on 2% agarose gels stained with ethidium bromide and the fragments were sized with 123 bp DNA ladder markers (Gibco BRL, Gaithersburg, MD, USA).

Results

Expected products of ~1 kb were obtained after PCR amplification of the PIB genes of all the 47 *N gonorrhoeae* strains tested. Six distinct PCR-RFLP profiles with *Cfo*I digestion (fig 1), and five different PCR-RFLP profiles with *MspA1*I digestion (fig 2) were observed. All the strains were tested at least three times, and the resultant PCR-RFLP patterns were consistent and reproducible.

Tables 1 and 2 diplay the distribution of the 47 serotyped strains according to PCR-RFLP patterns obtained after digestion with *Cfo*I and *MspA1*I, respectively. Strains belonging to serovars such as IB-2 and IB-3 could be further differentiated by *Cfo*I PCR-RFLP, each into four PCR-RFLP profiles. However, eight IB-7 and five IB-19 strains were clustered into *Cfo*I PCR-RFLP profile IV, while only two other IB-7 and one other IB-19 strains had different *Cfo*I profiles. Similarly, *MspA1*I PCR-RFLP also differentiated strains of the same serovar (for example IB-1 and IB-2) into distinct PCR-RFLP

Table 1 Classification of 47 N gonorrhoeae strains according to their respective CfoI PCR-RFLP profiles

PCR-RFLP profile	*Major fragments (bp)*	*Serovar*	*Strains*
I	420	IB-1	S7, S20
	345	IB-2	S2, S10
	255	IB-3	S31, S40, S52
		IB-4	S4
		IB-6	S27
		IB-14	S16
		IB-19	S3
II	690	IB-1	S24, S53, S55
	345	IB-2	S44, S58
		IB-3	S8, S33
		IB-5	S34
		IB-6	S18, S38, S51
III	640	IB-3	S12, S25
	345		
IV	690	IB-2	S5
	165	IB-3	S21
	105	IB-4	S11, S48
		IB-6	S17
		IB-7	S22, S26, S28, S30, S41, S43, S50, S57
		IB-19	S32, S35, S36, S37, S56
V	690	IB-2	S46
	255	IB-7	S1, S19
VI	430	IB-6	S39
	255	IB-9	S45

profiles. However, clustering of seven IB-3, five IB-6, six IB-7 and five IB-19 strains was observed.

The discriminatory power of auxotyping, serotyping and PCR-RFLP was compared (table 3). PCR-RFLP with both *Cfo*I and *MspA1*I could differentiate the 47 *N gonorrhoeae* isolates into 11 subtypes. Combined serovar/PCR-RFLP data were found to be more discriminatory than the auxotype/serovar typing system alone, yielding 23 and 21 subtypes with *Cfo*I and *MspA1*I digests, respectively; compared with only 17 auxotype/serovar classes among the 47 strains. When auxotyping, serotyping and PCR-RFLP analysis were combined, the strains could be further differentiated into 31 subtypes using *Cfo*I, or 28 subtypes using *MspA1*I. A total of 34 gonococcal subtypes could be differentiated when auxotyping and serotyping were combined with PCR-RFLP analyses using *Cfo*I and *MspA1*I.

Table 2 Classification of 47 N gonorrhoeae strains according to their respective MspA1I PCR-RFLP profiles

PCR-RFLP profile	*Major fragments (bp)*	*Serovar*	*Strains*
I	430	IB-1	S7, S20
	260	IB-2	S2, S10
	245	IB-3	S8, S12, S25, S31, S33, S40, S52
		IB-4	S4
		IB-5	S34
		IB-6	S18, S27, S38, S39, S51
		IB-9	S45
		IB-14	S16
II	430	IB-1	S53, S55
	325	IB-2	S58
	260		
III	515	IB-1	S24
	430	IB-2	S44, S46
		IB-7	S1, S19
		IB-19	S36
IV	595	IB-2	S5
	280	IB-3	S21
	160	IB-4	S11, S48
		IB-6	S17
		IB-7	S22, S26, S28, S30, S41, S43
		IB-19	S3, S32, S35, S37, S56
V	515	IB-7	S50, S57
	280		
	160		

Table 3 Comparison of auxotype, serovar and PCR-RFLP in differentiating 47 N gonorrhoeae strains

Classification method	*No of subtypes*
Auxotyping	2
Serotyping	10
PCR-RFLP (*Cfo*I)	6
PCR-RFLP (*MspA1*I)	5
PCR-RFLP (*Cfo*I)/PCR-RFLP (*MspA1*I)	11
Auxotype/Serovar	17
Auxotype/PCR-RFLP (*Cfo*I)	10
Auxotype/PCR-RFLP (*MspA1*I)	9
Serovar/PCR-RFLP (*Cfo*I)	23
Serovar/PCR-RFLP (*MspA1*I)	21
Auxotype/Serovar/PCR-RFLP (*Cfo*I)	31
Auxotype/Serovar/PCR-RFLP (*MspA1*I)	28
Auxotype/Serovar/PCR-RFLP (*Cfo*I)/PCR-RFLP (*MspA1*I)	34

Some strains, for example serovar IB-7 strains S26, S28, S30, S41 and S43 (Pro^-), could not be further differentiated by PCR-RFLP, analysis serotyping, auxotyping either individually or in combination.

Discussion

There are a number of limitations of serotyping. For example, some isolates cannot be serotyped by the standard panel of mAbs.[15] Moreover, serotyping requires specialised reagents, is expensive to maintain and is available only to a few reference centres. In contrast, all the strains in this study could be amplified by PCR as the primers were designed to anneal to the less variable termini of the PIB gene.[16] The PCR-RFLP method can also be used to study variation in *N gonorrhoeae* as it is not limited by the availability of existing or new mAbs.[14 15]

In this study, we found that PCR-RFLP can be used to differentiate strains belonging to PIB serovars, demonstrating its usefulness for inter- and intra-serotypic discrimination of *N gonorrhoeae* strains. With our PCR-RFLP method, strains belonging to a predominant serovar such as IB-3 could be further differentiated. Although IB-3 strains S12, S21, S31 and S33 could not be differentiated by auxotyping and serotyping, they could be distinguished from one another by PCR-RFLP analysis. PCR-RFLP could thus provide a higher degree of discrimination among gonococcal strains. Interestingly, IB-7 strains S26, S28, S30, S41 and S43 as well as IB-19 strains S32, S35 and S37 could not be further differentiated by PCR-RFLP, serotyping or auxotyping, suggesting that these strains may either be epidemiologically related or were derived from the same clone.

There was no absolute correlation between serotypes and PCR-RFLP profiles. Certain strains belonging to different serovars exhibited identical PCR-RFLP profiles, while some strains of the same serovar displayed different PCR-RFLP profiles. This may be attributed to the occurrence of restriction enzyme sites

outside of the regions responsible for encoding epitopes recognised by mAbs. Alternatively, silent or conservative nucleotide mutations could modify restriction sites without affecting epitope recognition by the mAbs.

From the published sequence of strain R10 (serovar IB-3) characterised in the USA,[13] predicted PCR-RFLP profiles with *Cfo*I (640 and 345 bp fragments) and *MspA1*I (430, 260, and 245 bp fragments) were found to be similar to those of strains S12 and S25 (also belonging to serovar IB-3). The PCR-RFLP profiles predicted from the published PIB sequences of strains S7, S11, S16, S22, S34 and S36 also correlated with the actual profiles on gel electrophoresis, thereby further supporting the reliability of our method. Our present study includes only 47 *N gonorrhoeae* strains belonging to 10 serovars. More PCR-RFLP profiles may be obtained with a larger number of strains belonging to the same or other serovars. In addition, the discriminatory power of PCR-RFLP analysis may be increased if more than two suitable enzymes were to be employed. It would be interesting to investigate whether this method can discriminate strains isolated from various geographical locations.

We have previously sequenced the hypervariable regions[17] of the gonococcal PIB genes of serovar IB-3, IB-7,[18] IB-4 and IB-5 strains.[9] The PCR-RFLP profiles were identical for serovar IB-3 strains S31, S40 and S52 which also shared identical nucleotide and predicted amino acid sequences of the hypervariable region of the PIB gene. PCR-RFLP analysis can be used as a preliminary screening method to type new gonococcal isolates prior to direct PCR sequencing which is more laborious and time-consuming. PCR-RFLP can be used as an adjunct to auxotyping, particularly due to the restricted number of auxotypes being encountered in a certain geographical area. Moreover, PCR-RFLP is rapid and easy to perform compared with ribotyping[19] and multilocus enzyme electrophoresis[20]. Thus, the PCR-RFLP method can augment existing typing methods such as auxotype/serovar classification to provide a higher degree of discrimination and for monitoring the spread of *N gonorrhoeae* in outbreak situations.

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