MOLECULAR CHARACTERISATION OF BACTERIA

Differentiation of *Neisseria gonorrhoeae* IB-3 and IB-7 serovars by direct sequencing of protein IB gene and pulsed-field gel electrophoresis

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Summary. Serotyping of *Neisseria gonorrhoeae* based on the differential recognition by a panel of six monoclonal antibodies (MAbs) against Protein IB (PIB) is a valuable tool for studying the epidemiology of gonorrhoea. However, the predominance of certain serovars in specific geographic regions necessitates the development of new MAbs or new genotyping approaches. Nucleotide and amino-acid sequence analysis of PIB from strains within the IB-3 and IB-7 serovars revealed strain differences within the same serovar. Based on the generation of PIB nucleotide and deduced amino-acid sequences that centred on amino-acid residues 196 and 237, eight serovar IB-3 strains and nine serovar IB-7 strains were separately subdivided into five groups. Intra-serovar differences were also established by pulsed-field gel electrophoresis (PFGE) of macro-restriction fragments generated by *SpeI*- and *NheI*-cleavage of genomic DNA. There was good correlation between the results based on PIB gene PCR-sequencing and those based on PFGE analysis of macro-restriction fragment patterns. These data demonstrate the high precision of PFGE analysis and indicate that this approach can be used as a rapid epidemiological subtyping system for major serovars of *N. gonorrhoeae*.

Introduction

Neisseria gonorrhoeae infection is one of the most common sexually transmitted diseases worldwide. Progress in vaccine development has been slow and the mainstay in the prevention of *N. gonorrhoeae* infection has been through epidemiological surveillance and antimicrobial treatment of infected individuals.¹ Epidemiological surveillance involves contact tracing, screening of high-risk, asymptomatic individuals in the community and the development of accurate strain characterisation markers to determine the spread of specific strains. The auxotype-serovar (A/S) classification is currently the most widely accepted method for the discrimination of *N. gonorrhoeae* isolates.² However, the A/S classification system has some major limitations because different strains often express the same auxotype.³ Serotyping provides a far more sensitive method for strain discrimination than auxotyping but there is scepticism that it may over-discriminate some serovars, while not providing sufficient discrimination of other strains within major serovars such as IB-1 and IB-3.⁴

Comparison of the entire nucleotide sequence of

gonococcal genomes may lead to a definitive typing method although this approach is relatively complex, expensive and impractical for large numbers of isolates. A simpler approach involves amplification, by the polymerase chain reaction (PCR), and direct DNA sequencing of a single gene, e.g., the *N. gonorrhoeae* protein IB (PIB) gene, which encodes serovar specificity.⁵ Alternatively, pulsed-field gel electrophoresis (PFGE) of gonococcal genomic DNA digested by low-frequency cleavage restriction endonucleases has been used to characterise *N. gonorrhoeae*.⁶ This approach examines the entire genome of isolates and differences in nucleotide sequences are reflected in the resulting DNA fingerprints. Since PFGE provided useful differentiation of common serovars in a previous study,⁶ the present study compared this method with PCR sequencing of the hypervariable region of the PIB gene for characterising *N. gonorrhoeae* strains.

Materials and methods

Bacterial isolates

Seventeen epidemiologically unrelated *N. gonor-*

a		550	560	570	580	590
IB3.S8		AACGGC	GAATCTTACC	ACGTTGGCTT	GAAGTACCAA	AACAGCGGCT
IB3.S33		*****	*****	***AA*****	*****	*****
IB3.S12		*****	*****	*****	*****	*****
IB3.S25		*****	*****	*****	*****	*****
IB3.S31		*****	*****	*****	*****	*****
IB3.S40		*****	*****	*****	*****	*****
IB3.S52		*****	*****	*****	*****	*****
IB3.S21		*****	*****	*****T**	*****G*	*****A*****
		600	610	620	630	640
IB3.S8		TCTTCGCACA	ATACGCCGGC	TTGTTCCAAA	GATACGGCGA	AGGCACTAAA
IB3.S33		*****	*****	*****	*****	*****
IB3.S12		*****	*****	*****	*****	*****
IB3.S25		*****	*****	*****	*****	*****
IB3.S31		*****	*****	*****	*****	*****
IB3.S40		*****	*****	*****	*****	*****
IB3.S52		*****	*****	*****	*****	*****
IB3.S21		*****	*****	*****	*****	*****
		650	660	670	680	690
IB3.S8		AAAATCGAAT	ACGATGGTCA	AGCTTATAGT	ATGCCCAGTC	TGTTTGTGTA
IB3.S33		*****	*****	*****	*****	*****
IB3.S12		*****	**A***A***	*****	**C*****C*	*****
IB3.S25		*****	**A***A***	*****	**C*****C*	*****
IB3.S31		*****	****ACA***	**T*****	**C*****C*	*****
IB3.S40		*****	****ACA***	**T*****	**C*****C*	*****
IB3.S52		*****	****ACA***	**T*****	**C*****C*	*****
IB3.S21		*****G***-	-----**AT*	T**A*****A*	**C*****T	*****
		700	710	720	730	740
IB3.S8		AAAATGCAA	GTTACCCGT	TGGTAGGCGG	TTACGACAAT	AATGCCCTGT
IB3.S33		*****	*****	*****	*****	*****
IB3.S12		*****	*****	*****	*****	*****
IB3.S25		*****	*****	*****	*****	*****
IB3.S31		*****	*****	*****	*****	*****
IB3.S40		*****	*****	*****	*****	*****
IB3.S52		*****	*****	*****	*****	*****
IB3.S21		*****	*****	***C*****	*****	*****
		750	760	770	780	788
IB3.S8		ACGTTTCCGT	AGCCGCACAA	CAACAAGATG	CCAAATTGTA	T--GGAGCAA
IB3.S33		*****	*****	*****	*****	*--*****
IB3.S12		*****	*****	*****	*****	*--*****
IB3.S25		*****	*****	*****	*****	*--*****
IB3.S31		*****	*****G***	*****	*****	*--*****
IB3.S40		*****	*****G***	*****	*****	*--*****
IB3.S52		*****	*****G***	*****	*****	*--*****
IB3.S21		***CC*****	*****	*****	*****	*CAAAT***
			804			
IB3.S8		CGAGGGT---	-TAATTCGCA	CAAC		
IB3.S33		*****---	*****	****		
IB3.S12		TA**C*G---	*****	****		
IB3.S25		TA**C*G---	*****	****		
IB3.S31		G*****C---	*****	****		
IB3.S40		G*****C---	*****	****		
IB3.S52		G*****C---	*****	****		
IB3.S21		AT*GTGCGTG	A*****	****		

Fig. 1. For legend see facing page.

rhoeae strains comprising eight IB-3 and nine IB-7 serovars were studied. These strains were part of a collection of 48 clinical strains previously isolated from patients attending a local hospital from June to August 1986.⁶ The strains were auxotyped and serotyped by Drs S. Sarafian and J. S. Knapp, Centers for Disease Control, Atlanta, GA, USA.⁴

PFGE

PFGE was performed with the contour-clamped homogeneous electric field apparatus (CHEF-DRII; BioRad, Richmond, CA, USA) as described previously.⁶ Briefly, genomic DNA samples in agarose plugs (In Cert Agarose; FMC, Rockland, ME, USA)

b

	160	170	180	190	aa aa a ## ## #
IB3.S8	NGES	YHVGLNYQNS	GFFAQYAGLF	QRYGEGTKKI	EYDGGAYSMP
IB3.S33	****	**E*****	*****	*****	*****
IB3.S12	****	*****	*****	*****	**ND****I*
IB3.S25	****	*****	*****	*****	**ND****I*
IB3.S31	****	*****	*****	*****	**EH*V**I*
IB3.S40	****	*****	*****	*****	**EH*V**I*
IB3.S52	****	*****	*****	*****	**EH*V**I*
IB3.S21	****	*****R*N	*****	*****M	*--*Y**NI*

	210	220	230	hhhhh #####	h #
IB3.S8	SLFVEKLQVH	RLVGGYDNNA	LYVSVAAQQQ	DAKLYGAT--	RVNSHN
IB3.S33	*****	*****	*****	*****--	*****
IB3.S12	*****	*****	*****	*****I--	SG****
IB3.S25	*****	*****	*****	*****I--	SG****
IB3.S31	*****	*****	*****	*****R--	*A****
IB3.S40	*****	*****	*****	*****R--	*A****
IB3.S52	*****	*****	*****	*****R--	*A****
IB3.S21	*****	*****	**A*****	*****QNQIV	*D****

Fig. 1. a, Comparative nucleotide sequence analysis of PIB gene fragments of serovar IB-3 strains. The numbering is according to Butt *et al.*⁷ *: identity; -: deletion. b, Comparison between deduced amino-acid sequences of the protein IB⁸ of serovar IB-3 strains. *: identity; -: deletion; a: recognised by MAb 3C8; h: recognised by MAb 2D4.

were digested with *SpeI* or *NheI* (New England Biolabs, Beverly, MA, USA) overnight at 37°C in 150 µl of restriction endonuclease buffer containing 10 U of endonuclease. Digested plugs were electrophoresed in agarose 1% gels (SeaKem GTG agarose, FMC) for 22 h with pulse times of 1–15 s. Gels were stained with ethidium bromide, destained in water, and photographed under UV illumination.

PCR and direct cycle sequencing

Extraction of bacterial DNA and PCR amplification of a 341-bp fragment (nucleotides 519–859) of the variable region of the *N. gonorrhoeae* PIB gene with OMG1 and OMG2 primers were as described previously.⁵ The PCR products were purified and their DNA sequences were determined in both directions by direct cycle sequencing with ³²P-labelled OMG1 and OMG2 primers as sequencing primers, as described previously.⁵ The resultant nucleotide sequences were converted to their respective amino-acid sequences by the DNASIS program, and then aligned for maximum homology with the PROSIS program (Hitachi, Brisbane, CA, USA).

Results

Nucleotide and deduced amino-acid sequences of PIB-3 and PIB-7 serovar strains

Comparison of the nucleotide sequences of the eight IB-3 strains revealed diversity centred around two discrete regions corresponding to nucleotides 646–680 and 782–795 (fig. 1a).⁷ Analysis of the deduced amino-acid sequences of the eight strains indicated changes within codons 190–199 and 236–240 (fig. 1b).⁸ Comparison of nucleotide sequences of the nine IB-7 strains

also indicated differences mainly at positions 646–680 (fig. 2a) corresponding to amino-acid changes at codons 190–199 (fig. 2b).

Comparing the DNA sequences of the eight IB-3 strains showed that there were 2–36 nucleotide differences. Of the maximum number of 36 nucleotide changes, six were silent. Strains S8 and S33 differed in only two nucleotides whereas strain S21 differed from strains S31, S40 and S52 at 36 nucleotide positions. The deduced changes in amino-acid residues varied from a single difference (between strains S8 and S33) up to 16 differences (between strains S21 and S33). The eight IB-3 strains were subdivided into five groups based on nucleotide and amino-acid sequences. The distribution of strains into their respective groups based on nucleotide sequences was found to concur with that based on amino-acid sequences (table I).

Amongst the nine IB-7 strains, only two nucleotide differences were observed between strains S22 and S43; the highest number of 27 nucleotide differences was found between strains S1 and S43, five of these being silent changes. Comparison of deduced amino-acid residues of the nine IB-7 strains revealed from two amino-acid changes (between strains S22 and S43) up to 13 changes (between strains S1 and S19). Based on nucleotide and deduced amino-acid sequences, the nine IB-7 strains were subdivided into five groups. With the exception of a single group comprising five strains, the remaining four groups each consisted of a single isolate. There was perfect correlation between the grouping of IB-7 strains based on nucleotide and amino acid sequences (table II).

PFGE

Restriction endonucleases *SpeI* and *NheI* were found to generate PFGE profiles that were easily read.

a					
	550	560	570	580	590
IB7.S43	...AACGGC	GAATCTTACC	ACGTTGGTTT	GAACTACCGA	AACAACGGCT
IB7.S22	...*****	*****	*****	*****	*****
IB7.S26	...*****	*****	*****C**	*****	*****
IB7.S28	...*****	*****	*****C**	*****	*****
IB7.S30	...*****	*****	*****C**	*****	*****
IB7.S41	...*****	*****	*****C**	*****	*****
IB7.S50	...*****	*****	*****C**	*****	*****
IB7.S19	...*****	*****	*****C**	*****A*	****G*****
IB7.S1	...*****	*****	*****C**	*****A*	****G*****
	600	610	620	630	640
IB7.S43	TCTTCGCACA	ATACGCCGGC	TTGTTCCAAA	GATACGGCGA	AGGCACTAAA
IB7.S22	*****	*****	*****	*****	*****
IB7.S26	*****	*****	*****	*****	*****
IB7.S28	*****	*****	*****	*****	*****
IB7.S30	*****	*****	*****	*****	*****
IB7.S41	*****	*****	*****	*****	*****
IB7.S50	*****	*****	*****	*****	*****
IB7.S19	*****	*****	*****	*****	*****
IB7.S1	*****	*****	*****	*****	*****
			670	680	690
IB7.S43	AAAATCGAA-	-----GGCTA	TCAGTATAAT	AGCCCCAGTC	TGTTTGTGTA
IB7.S22	*****-	-----**C*	*****	*T*****	*****
IB7.S26	*****G***-	-----**A**	*ACA*****	*T*****T	*****
IB7.S28	*****G***-	-----**A**	*ACA*****	*T*****T	*****
IB7.S30	*****G***-	-----**A**	*ACA*****	*T*****T	*****
IB7.S41	*****G***-	-----**A**	*ACA*****	*T*****T	*****
IB7.S50	*****G***-	-----**A**	*ACA*****	*T*****T	*****
IB7.S19	*****G***-	-GGAT-AT**	*TTA*****	*T*****T	*****
IB7.S1	*****T	ACGAT**TC*	AGCT***G*	*TG*****	*****
	700	710	720	730	740
IB7.S43	AAAAC TGCA	GTTCACCGTT	TGGTCGGCGG	TTACGACAA	AATGCCCTGT
IB7.S22	*****	*****	*****	*****	*****
IB7.S26	*****	*****	*****	*****	*****
IB7.S28	*****	*****	*****	*****	*****
IB7.S30	*****	*****	*****	*****	*****
IB7.S41	*****	*****	*****	*****	*****
IB7.S50	*****	*****	*****	*****	*****
IB7.S19	*****	*****	*****	*****	*****
IB7.S1	*****	*****	*****A*****	*****	*****
	750	760	770	780	790
IB7.S43	ACGCCTCCGT	AGCCGCACAA	CAACAAGATG	CCAAATTGTA	TGGAACATGG
IB7.S22	*****	*****	*****	*****	*****
IB7.S26	*****	*****	*****	*****	*****
IB7.S28	*****	*****	*****	*****	*****
IB7.S30	*****	*****	*****	*****	*****
IB7.S41	*****	*****	*****	*****	*****
IB7.S50	*****	*****	*****	*****	*****
IB7.S19	*****	*****	*****	*****	*****
IB7.S1	***TT*****	*****	*****	*****	****G**AC*
	800	810	820	830	
IB7.S43	CGTGCTAATT	CGCACAACTC	TCAAACCGAA	GTTGCCGCTA	CC
IB7.S22	*****	*****	*****	*****	**
IB7.S26	*****	*****	*****	*****	**
IB7.S28	*****	*****	*****	*****	**
IB7.S30	*****	*****	*****	*****	**
IB7.S41	*****	*****	*****	*****	**
IB7.S50	*****	*****	*****	*****	**
IB7.S19	*A*****	*****	*****	*****	**
IB7.S1	A*G*T*****	*****	*****	*****	**

Fig. 2. For legend see facing page.

b

	160	170	180	190	aa aa a ## ## #
IB7.S43	NGES	YHVGLNYRNN	GFFAQYAGLF	QRYGEGTKKI	E--GYQYNSP
IB7.S22	****	*****	*****	*****	*--H***I*
IB7.S26	****	*****	*****	*****M	*--***T**I*
IB7.S28	****	*****	*****	*****M	*--***T**I*
IB7.S30	****	*****	*****	*****M	*--***T**I*
IB7.S41	****	*****	*****	*****M	*--***T**I*
IB7.S50	****	*****	*****	*****M	*--***T**I*
IB7.S19	****	*****Q*S	*****	*****M	*-GY*L**I*
IB7.S1	****	*****Q*S	*****	*****	*YD*QA*SM*

	210	220	230	hhhhh #####	h #	248
IB7.S43	SLFVEKLQVH	RLVGGYDNNA	LYASVAAQQQ	DAKLYGTW--	RANSHNSQTE	VAAT
IB7.S22	*****	*****	*****	*****--	*****	****
IB7.S26	*****	*****	*****	*****--	*****	****
IB7.S28	*****	*****	*****	*****--	*****	****
IB7.S30	*****	*****	*****	*****--	*****	****
IB7.S41	*****	*****	*****	*****--	*****	****
IB7.S50	*****	*****	*****	*****--	*****	****
IB7.S19	*****	*****	*****	*****--	H*****	****
IB7.S1	*****	*****	**V*****	*****AT--	*V*****	****

Fig. 2. a, Comparative nucleotide sequence analysis of PIB gene fragments of serovar IB-7 strains. *: identity; --: deletion. b, Comparison between deduced amino-acid sequences of the protein IB of serovar IB-7 strains. *: identity; --: deletion; a: recognised by MAb 3C8; h: recognised by MAb 2D4.

Table I. *N. gonorrhoeae* IB-3 serovar strains analysed by auxotyping, PFGE of *SpeI* and *NheI* macro-restriction fragments and PIB nucleotide and amino-acid sequences

Strain no.	Auxotype	<i>SpeI</i> pattern	<i>NheI</i> pattern	Nucleotide sequence pattern	Amino-acid sequence pattern	GenBank no.
S8	Proline	1	1	1	1	U17238
S40	Proline	2	2	2	2	U17029
S12	Prototrophic	3	3	3	3	U17020
S21	Prototrophic	4	4	4	4	U17022
S25	Prototrophic	3	3	3	3	U17023
S31	Prototrophic	2	5	2	2	U17027
S33	Prototrophic	5	6	5	5	U17028
S52	Prototrophic	6	7	2	2	U17237

Table II. *N. gonorrhoeae* IB-7 serovar strains analysed by auxotyping, PFGE of *SpeI* and *NheI* macro-restriction fragments and PIB nucleotide and amino-acid sequences

Strain no.	Auxotype	<i>SpeI</i> pattern	<i>NheI</i> pattern	Nucleotide sequence pattern	Amino-acid sequence pattern	GenBank no.
S1	Proline	1	1	1	1	U17019
S19	Proline	2	2	2	2	U17021
S26	Proline	3	3	3	3	U17024
S28	Proline	3	3	3	3	U17025
S30	Proline	3	3	3	3	U17026
S41	Proline	3	3	3	3	U17234
S43	Proline	4	4	4	4	U17235
S22	Prototrophic	5	5	5	5	U09044
S50	Prototrophic	6	6	3	3	U17236

As shown in fig. 3a, each *SpeI* fingerprint of IB-3 strains contained 13–17 fragments, ranging from 2 to 350 kb. Digestion with *NheI* produced 14–16 fragments, ranging from 20 to 410 kb.⁶ *SpeI* and *NheI* digestion of genomic DNA samples from the eight

IB-3 strains revealed six and seven DNA patterns, respectively (table I). Amongst the IB-7 strains, *SpeI* and *NheI* analysis each generated six patterns (fig. 3b),⁶ and the distribution of strains into their respective patterns correlated highly (table II).

Fig. 3. PFGE analysis of *SpeI*-cleaved genomic DNA samples of *N. gonorrhoeae*: a, IB-3 strains; b, IB-7 strains. The strain numbers are given above each track. *Rhodobacter sphaeroides* 2.4.1 digested with *AseI* served as the size marker (track 1).

Discussion

PFGE resolution of chromosomal macro-restriction fragments has been shown to be a highly discriminatory tool for the delineation of genetic relatedness of bacterial pathogens.⁹⁻¹¹ In the present study, PFGE analysis of *SpeI* and *NheI* macro-restriction fragments generally correlated well with direct PCR sequencing of PIB gene, albeit with some discrepancies. For example, the IB-3 serovar strains S40, S31 and S52 showed identical nucleotide and amino-acid sequences but the three strains were subtyped into two *SpeI* and three *NheI* patterns. Of the nine strains belonging to serovar IB-7, four strains (S26, S28, S30 and S41) were found to have identical PFGE profiles, as well as nucleotide and deduced amino-acid sequences. The only discordance was found with strain S50 which was in the same nucleotide and amino-acid group as strains S26, S28, S30 and S41 but exhibited both *SpeI* and *NheI* PFGE patterns that differed significantly from these strains. The occurrence of different PFGE profiles in strains having identical PIB nucleotide and amino-acid sequences could be explained by the divergence in nucleotide sequences at other sites of the genome through either point mutations or genome rearrangements. If such genetic events were to occur in the PIB gene itself, and if the mutations were to affect the overall epitope conformation, serovar changes could be expected. An alternative possibility, especially in a naturally transformable organism like *N. gonorrhoeae*, could be that this PIB gene may have been acquired by strain S50 by genetic recombination with the PIB sequence of a group 3 strain. The PIB gene analysis of 1 kb of DNA represents only c. 0.1% of the bacterial genome and differences outside this

region cannot be detected. In contrast, PFGE examines the entire genome and can, therefore, potentially detect more variation.

Comparison of the amino-acid sequences of all IB-3 strains, which were recognised by a panel of monoclonal antibodies (MAbs) comprising MAbs 3C8 ('a'), 1F5 ('b') and 2H1 ('k'), and all IB-7 strains, which were recognised by a panel of MAbs comprising 2D6 ('c'), 2G2 ('g') and 2H1 ('k'),⁴ indicated that the results from the present study concur with those from our previous study¹² in which we proposed that codons 192, 193, 195, 196 and 198 were responsible for epitope recognition by MAb 3C8 ('a'). Changes in 1-4 of these amino acids did not prevent recognition by MAb 3C8 ('a'). In contrast to the other IB-3 strains, S21 had glutamine, asparagine, glutamine, isoleucine, valine and aspartic acid at positions 236-240. These positions have been proposed to be the epitope recognised by MAb 2D4 ('h'), a MAb which does not recognise serovar IB-3 strains.¹² A discrepancy thus emerged since strain S21 did not react with MAb 2D4 and hence was typed as serovar IB-3. Furthermore, strain S1 had amino-acid residues tyrosine, aspartic acid, glutamine, alanine and serine at positions 192, 193, 195, 196 and 198, respectively, corresponding to the epitope for MAb 3C8¹² but was typed as serovar IB-7 which should not react with MAb 3C8. These two discrepancies could be due to the subjective nature of interpretation of co-agglutination results from weak reactions or could have arisen from conformational hindrance caused by reactive epitopes, thereby preventing optimal recognition and binding of the MAbs.

The present study showed that despite the majority of strains being grouped within the same serovar, they could still be differentiated on the basis of PIB

nucleotide and amino-acid sequence differences, especially at the epitope regions. The high correlation observed between PFGE, nucleotide and deduced amino-acid sequence analysis indicated that PFGE is a highly discriminatory tool, which may be more suitable for strain characterisation of *N. gonorrhoeae* for

epidemiological studies than direct PCR sequencing of the protein IB gene.

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