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Genetic relationships among *Neisseria gonorrhoeae* serovars analysed by multilocus enzyme electrophoresis

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SUMMARY

Multilocus enzyme electrophoretic analysis was employed to assess the genetic relatedness of *Neisseria gonorrhoeae*. Based on the diversity of electromorphs at 9 enzyme loci, 16 electrophoretic types (ETs) were established amongst the 65 isolates. The average number of alleles per enzyme locus was 1·7 and the mean genetic diversity per locus was 0·212. The majority of isolates belonged to either ET1 (32·3%) or ET2 (16·9%). No specific correlation of ETs was seen with serovars as the major types, ETs 1 and 2, were found distributed amongst the various serovars. Major serovars such as Bacjk (IB-1/2) and Bajk (IB-3/6) were each represented by 6 or 8 ETs respectively. Analysis of the genetic relationships of ETs to each other showed some clustering of subgroups that were more closely related than others.

INTRODUCTION

Serological methods based on the antigenic determinants of protein IA or IB have been used effectively to differentiate *Neisseria gonorrhoeae* into three groups designated WI, WII and WIII [1]. Development of monoclonal antibodies directed against epitopes on gonococcal outer membrane protein I molecules by Tam and colleagues [2] facilitated further serological resolution among *N. gonorrhoeae* strains. Strains have been assigned to serovars by their patterns of reactivity with six protein IA-specific and six protein IB-specific monoclonal antibody reagents [3, 4]. These highly specific monoclonal antibodies have been used as probes to detect antigenic variation of serovar strains. However, the genetic variation between strains of identical serovar or between strains belonging to different serovars has not been examined.

Recently, the technique of multilocus enzyme electrophoresis has been applied to study the genetic variation of a variety of bacteria [5]. The method consists of measuring allelic variation at multiple structural gene loci by screening randomly selected cytoplasmic enzymes for genetically controlled variants (electromorphs or allozymes). 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 objectives of the present study were to evaluate the application of multilocus enzyme electrophoresis to differentiate *N. gonorrhoeae* serovars and to assess the genetic relationships among them.

MATERIALS AND METHODS

Bacterial strains

A total of 65 *Neisseria gonorrhoeae* strains were randomly isolated for a period of 6 months from male and female patients in Singapore.

Serological classifications

The gonococcal strains were characterized serologically with specific monoclonal antibody reagents against outer membrane proteins I A and I B (Table 1). Protein I A-specific antibodies used were designated as follows: 4A12, b; 2F12, d; 6G9, f; 6D9, g; 5D1, h; 5G9, i; and 5C2, k. Identification of W II and W III strains was achieved by using eight protein I B-specific monoclonal antibodies. Protein I B-specific antibodies were designated as follows: 3C8, a; 1F5, b; 2D6, c; 2H7, e; 2G2, g; 2D4, h; 3B10, j; and 2H1, k. All the designations of the W I serovars begin with an A (protein I A), and the designations of the W II and W III serovars begin with a B (protein I B).

Preparation of bacterial extracts for electrophoresis

Each isolate was streaked onto ten modified Thayer Martin (MTM) agar plates. After overnight incubation in a CO_2 incubator, the cells were harvested with 2 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 6·8) from each plate, pooled and centrifuged at 8000 rev./min for 20 min. The pellet was washed with the same buffer once. After centrifugation, the pellet was resuspended in 3 ml of TE buffer. The cells were then lysed by sonication using the Braun cell disrupter (Model 30) four times using 30 s bursts with intermittent cooling on an ice-bath. The crude extracts were centrifuged at 16000 g for 30 min and the clear supernatants used immediately.

Electrophoresis of enzymes

Electrophoresis of extracts was performed on polyacrylamide gels using the Davis system of discontinuous gels [6]. Homogenous (7% and 10%) gels as well as gradient gels (5–15%) were used; the system chosen depended on the enzyme to be screened. Running conditions for all electrophoresis was performed at a constant voltage of 50 V for 17 h for 7% polyacrylamide gels, 60 V for 19 h for 10% and gradient (5–15%) polyacrylamide gels.

Specific enzyme staining

Enzymes present in each extract were assayed according to the methods described by Selander and co-workers [5]. Nine enzymes were stained: alkaline phosphatase (ALP), β-esterase (EST), $NADP^+$-dependent glutamate dehydrogenase (GD), glucose dehydrogenase (GDH), glucose-6-phosphate dehydrogenase (G6PD), isocitrate dehydrogenase (IDH), indophenol oxidase (IPO), leucine aminopeptidase (LAP) and phosphoglucose isomerase (PGI).

For each enzyme, distinctive mobility variants (electromorphs), numbered in order of their decreasing rate of anodal migration, were equated with alleles at the corresponding structural gene locus. Each isolate was characterized by its combination of alleles over the nine gene loci assayed. Distinctive combinations of

Table 1. *Serological characteristics of 65* Neisseria gonorrhoeae *strains*

Gonococcal serovar	Designation of Knapp and co-workers [4]	Strain
Aed	IA-8	D28
Aedih	IA-6	D20, D48, D53, D55, D59, S44
Bacjk	IB-1/2	D1, D25, D36, D4, D42, D47, D50, D51, D67, D68, S20, S3, S8
Back	IB-2	D21, D34, D35, D7, D71
Bajk	IB-3/6	D12, D14, D15, D23, D27, D3, D37, S28, S61, S7
Bak	IB-6	D18, D26, S31
Bcegjk	IB-5/7	D17, D5, D57
Bcegk	IB-5/7	S40
Bcgjk	IB-5/7	D32, S1, S23, S41, S58, S9
Bcgk	IB-7	D10, D33, D52, S14, S33
Bck	IB-19	D30, D6, D9
Bk	IB-32	D24
Beghjk	IB-4/11	D31, D54, D58, D65, S11, S42
BhK	IB-15	S64

alleles, corresponding to unique multilocus genotypes, were designated as electrophoretic types (ETs).

Statistical analysis

Genetic diversity at an enzyme locus (h) among ETs or isolates was calculated from allele frequencies as $H = (1-EX_i^2)[n/n-1)$, where X_i is the frequency of the ith allele and n is the number of Ets or isolates [7]. Mean genetic diversity per locus (H) is the arithmetic average of h values for all loci. Genetic distance between pairs of ETs was expressed as the proportion of enzyme loci at which different alleles were represented (mismatches).

Numerical classification

Genetic relationships between *N. gonorrhoeae* serovars were determined using a dendrogram based on the commercial program NTSYS-pc [8].

RESULTS

Enzyme locus variation

Only 9 of the 19 enzymes tested were selected for further study. The other 10 enzymes were either present at very low levels in all the strains – for example, malate dehydrogenase (MDH), malic enzyme (ME) and phosphoglucomutase (PGM) – or were not reproducible, for example, aspartate dehydrogenase (ASD), 3-hydroxybutyrate dehydrogenase (HBD) and xanthine dehydrogenase (XDH). With the exception of β-esterases which were encoded by two gene loci, the remaining enzymes were all encoded by a single locus. The gene locus encoding for the more anodal, faster-moving EST was designated EST-1 while the slower-moving less-anodal EST was encoded by the EST-2 locus. Both loci were polymorphic. Indophenol oxidase (IPO), glucose-6-phosphate dehydrogenase (G6PD) and phosphoglucose isomerase (PGI) were the only monomorphic enzymes

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Table 2. *Genetic diversity of ten enzyme loci among 65 isolate of* Neisseria gonorrhoeae

Enzyme locus	No. of alleles	Genetic diversity
Alkaline phosphatase	2	0·227
Esterase 1	2	0·435
Esterase 2	2	0·367
Glutamate dehydrogenase	2	0·227
Glucose dehydrogenase	2	0·227
Glucose-6-phosphate dehydrogenase	1	0
Isocitrate dehydrogenase	2	0·227
Indophenol oxidase	1	0
Leucine aminopeptidase	2	0·410
Phosphoglucose isomerase	1	0

The mean number of alleles was 1·7. The mean genetic diversity ($\bar{n}$) was 0·212 for isolates.

Table 3. *Allele profiles for 10 enzyme loci in 16 ETs of* Neisseria gonorrhoeae

		Allele at the indicated enzyme locus									
ET	No. of strains	ALP	EST-1	EST-2	GD	GDH	G6P	IDH	IPO	LAP	PGI
1	21	2	0	1	2	1	1	1	1	1	1
2	11	2	0	1	2	1	1	1	1	2	1
3	9	2	0	1	2	0	1	1	1	2	1
4	5	2	1	1	2	1	1	1	1	1	1
5	5	2	1	0	2	1	1	1	1	1	1
6	2	2	1	1	2	1	1	1	1	2	1
7	2	1	1	0	2	1	1	1	1	1	1
8	2	2	0	1	2	1	1	1	1	1	1
9	1	2	1	1	2	1	1	2	1	1	1
10	1	2	1	0	2	1	1	2	1	1	1
11	1	2	0	1	1	0	1	1	1	2	1
12	1	2	0	1	2	1	1	2	1	1	1
13	1	2	0	1	1	1	1	1	1	1	1
14	1	2	1	0	1	1	1	1	1	1	1
15	1	1	1	0	2	1	1	1	1	2	1
16	1	2	1	1	2	0	1	1	1	2	1

ALP, Alkaline phosphatase; EST-1, esterase 1; EST-2, esterase 2; GD, glutamate dehydrogenase; GDH, glucose dehydrogenase; G6P, glucose-6-phosphate dehydrogenase; IDH, isocitrate dehydrogenase; IPO, indophenol oxidase; LAP, leucine aminopeptidase; PGI, phosphoglucose isomerase.

amongst the nine enzymes examined. The polymorphic enzymes each had two alleles. Genetic diversity at each enzyme locus was found to range from 0·227 to 0·435 with a mean genetic diversity per locus of 0·212 (Table 2). The EST-1 locus displayed the highest genetic diversity value of 0·435. A comparison of the allele profiles of the 65 strains showed that 16 distinct allele combinations or ETs were present (Table 3). Eight of the 16 ETs were represented by single isolates while the remaining were represented by two or more isolates. ET1 was predominant, representing 32·3% of the total number of isolates. Two ETs (ET2 and ET3) were found amongst 17% and 13·8% of the isolates respectively.

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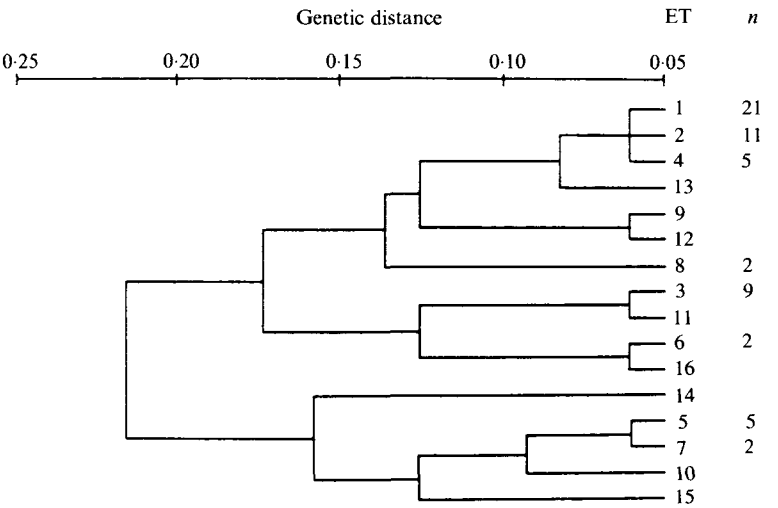

Fig. 1. Genetic relationships among the 16 ETs of *Neisseria gonorrhoeae*. The dendogram was generated by the average-linkage method of clustering from a matrix of coefficients of pairwise genetic distance based on ten enzyme loci. The number of isolates (*n*) in each ET represented by multiple isolates is indicated.

Relationships among ETs

The genetic relationships among the 16 ETs is shown in the dendrogram in Fig. 1. The smallest genetic distance (0·062) observed corresponds to a single locus difference while the largest distance (0·375) corresponds to differences at six loci. There were two clusters of ETs branching at or above a genetic distance of 0·187, which reflects differences between clusters at three enzyme loci. One cluster comprised 11 ETs while the other 5 ETs. The two clusters could be further subdivided at a genetic distance of 0·125, which indicated differences at two enzyme loci. At this genetic distance, the larger cluster was further resolved into five subgroups while the smaller cluster was divided into three subgroups.

Relationship between ET and serovar

Variation in serovar patterns were found among isolates belonging to the same ET. As shown in Table 4, ET-1 included 21 strains comprising 11 serovars. Similarly, ET-2 to -8 were also composed of multiple serovars. Out of the 16 ETs, only 8 ETs (ET-9 to -16) were each associated with a single serovar.

Genetic variation among serovars

Based on analysis of variability at 10 enzyme loci, the 14 serovars used in this study could be subdivided into 16 ETs. Major serovars such as Bajk (IB-3/6), Bacjk (IB-1/2) and Beghjk (IB-4/11) were represented by isolates belonging to several ETs. There were 8 ETs in serovar Bajk, 6 in serovar Bacjk and 5 in serovar Beghjk. Serovars Bcgk (IB-7) and Bcgjk (IB-5/7) were each represented by isolates of 4 ETs; serovars Aedih (IA-6), Bak (IB-6), Bck (IB-2) and Becgjk (IB-5/7) each included isolates of 3 ETs; and serovar Bck (IB-19) was represented by 2 ETs. Serovars Aed (IA-8), Bcegk (IB-5/7), Bk (IB-32) and Bhk (IB-15) were the only serovars with single ETs.

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Table 4. *Genetic variation among serovars of* Neisseria gonorrhoeae

Serovar	ET	No. of strains	Total no. of ETs	Serovar	ET	No. of strains	Total no. of ETs
Aed	1	1	1	Bcegjk	2	1	3
					7	1	
Aedih	1	2	3		13	1	
	2	3					
	4	1		Bcegk	3	1	1
Bacjk	1	4	6	Bcgjk	1	3	4
	2	2			3	2	
	3	3			8	1	
	4	1			11	1	
	5	2					
	14	1					
Back	2	3	3	Begk	1	2	4
	5	1			3	1	
	9	1			5	1	
					8	1	
Bajk	1	2	8	Bck	1	2	2
	2	1			3	1	
	3	1					
	4	2		Bk	1	1	1
	5	1					
	6	1		Beghjk	1	2	5
	7	1			2	1	
	16	1			4	1	
					6	1	
					15	1	
Bak	1	1	3				
	10	1					
	12	1		Bhk	1	1	1

DISCUSSION

In the present study, multilocus enzyme electrophoresis revealed significant genetic heterogeneity among strains belonging to the same serovar. The results showed that 16 ETs (multilocus genotypes) could be established amongst the 65 isolates representing 14 serovars. Ten strains of serovar Bajk (1B-3/6) were further resolved into eight ETs. Conversely, the major electrophoretic type, ET1, was found to be comprised of 11 serovars. Thus, identity in a single phenotypic character like serovar can be grossly misleading as to the genetic relationships of organisms. Recent studies have also revealed that clonal relationships among *Escherichia coli* [9] and *Neisseria meningitidis* [10] are not necessarily reflected by identity of serotype.

If clones are defined by multilocus enzyme genotype, as it indexes the chromosomal genome, there arises the problem of whether to define each ET as a clone with complete genotypic identity or to adopt a less-rigorous criterion. As a working rule, it has been recommended by Selander and colleagues [9] that isolates that differed at no more than two or three loci be treated as the same clone. By the definition suggested, taking a genetic distance of 0·125, which indicated differences at two enzyme loci (Fig. 1), the 65 isolates can be taken as being comprised of 8 clones. Two of these were composed of more subclones.

Our present analysis indicated the close genetic relatedness of ETs that are found within clusters separating at a genetic distance of 0·125. ETs 1, 2, 4 and 13 can be taken as subclones derived from the same ancestral clone. Thus, serovars found within these four ETs are closely related. The evolution of serovars can then be more precisely defined. The finding of ET1 being represented by several serovars indicated the evolution of subclones expressing different epitopes from a single common clone. These subclones could have arisen either through mutation or recombination.

The majority of isolates were found to belong to either ET1 (32·3%) or ET2 (16·9%) and the two ETs comprised 49·2% of the isolates under study. A higher degree of heterogeneity was observed among the strains when differentiation was based on the auxotype/serological (A/S) classification than multilocus enzyme electrophoretic analysis [11, in press]. Caugant and co-workers [10] similarly found little relationship between serogroups, serotypes and clonal genetic structure of *N. meningitidis* as revealed by enzyme electrophoresis. In their study, 87% of the total species diversity was represented by different ETs of the same serotype. If this is indeed the case for *N. gonorrhoeae*, epidemiological interpretation of outbreaks may take on a different picture to that presented by serotyping.

The absence of correlation between multilocus genotypes and serotypes suggests that it may be possible to combine both approaches to differentiate *N. gonorrhoeae* isolates. Our data showed the ability of multilocus enzyme electrophoretic analysis to further subtype common serotypes. Utilization of the combined approach would entail the designation of strains by serotype and an ET number, for example, type Bacjk (1) represented serotype Bacjk and ET1.

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