Subtyping of *Neisseria gonorrhoeae* auxotype-serovar groups by pulsed-field gel electrophoresis

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Summary. Genomic DNA from 48 *Neisseria gonorrhoeae* isolates was digested with low-frequency cleavage (LFC) endonucleases (*SpeI* and *NheI*) and analysed by contour-clamped homogeneous electric fields electrophoresis (CHEF). The restriction patterns generated were reproducible, stable, easy to read and offer a more practical approach than restriction endonuclease analysis (REA) with high-frequency cleavage (HFC) endonucleases. Strains sharing common auxotypes and serovars could be differentiated and correlation with auxotype/serovar (A/S) classes was demonstrated for some, but not all strains. The strains were distributed into 18 A/S classes and 38 *SpeI* and 40 *NheI* restriction patterns. This greater discriminatory power of CHEF REA should allow subdivision of strains within common A/S classes.

Introduction

The use of various typing schemes has greatly facilitated understanding of the molecular epidemiology of *Neisseria gonorrhoeae*.¹ Serotyping with monoclonal antibodies (MAbs) has allowed greater differentiation of strains than either auxotyping or plasmid-profile analysis.² However, serotyping has two major limitations; new MAbs have to be developed continually to type strains not covered by the existing panel and more discrimination is required within major serovars such as IB-1 and IB-3.³ Some strains react with only one MAb and if these strains were to mutate, they would no longer be typeable.⁴ Other problems include the long-term availability of MAbs, batch-to-batch reagent variation and the reproducibility of co-agglutination reactions, especially with MAbs 2D6, 2G2 and 6D9.⁵ The auxotype-serovar (A/S) classification system is currently the most widely accepted method for typing *N. gonorrhoeae* despite the inherent limitations of its component systems.

In restriction endonuclease digest analysis (REA) resolved by conventional polyacrylamide⁶ or agarose gel⁷ electrophoresis, no strong correlation was found between REA patterns and serovar classification. However, REA often produces complex patterns that are difficult to interpret. Other variables affecting REA patterns are the choice of restriction endonucleases used and the electrophoresis systems employed.

Genotypic characterisation of strains by pulsed-field gel electrophoresis (PFGE) has been found to be useful for analysis of patterns generated by restriction

enzymes that cut chromosomal DNA infrequently.⁸ A small number of high-mol.-wt DNA fragments are produced, giving simple, readily interpretable patterns. Furthermore, electrophoresis conditions can be adjusted to resolve fragments over a wide range of molecular sizes, enabling the whole genome to be analysed on a single gel. PFGE has been found to be highly discriminatory in studies of *Candida albicans*,⁹ *Pseudomonas aeruginosa*,¹⁰ *Staphylococcus aureus*,¹¹ *Campylobacter jejuni*¹² and *Escherichia coli*.¹³

This study describes the application of PFGE for typing of *N. gonorrhoeae* in comparison with A/S typing.

Materials and methods

Bacterial strains and culture conditions

Forty-eight clinical isolates of *N. gonorrhoeae* (table) were grown on modified Thayer-Martin Agar (MTM; BBL) and incubated at 37°C in CO₂ humidified air with CO₂ 5% for 20 h.

Serological characterisation

Strains were serotyped by S. Sarafian and J. Knapp, Centers for Disease Control, Atlanta, GA, USA, with six MAbs specific for protein IA (PrIA) and six specific for protein IB (PrIB).

Genomic DNA preparation

Fresh overnight cultures were harvested, washed with PIV solution (10 mM Tris-HCl, pH 7.5, 1 M NaCl), resuspended in 2 ml of PIV, mixed with 2 ml of low-melting point agarose (Sea Plaque LMP; FMC Corp., Marine Colloids Div., Rockland, ME, USA)

Table. Phenotypic characteristics of *N. gonorrhoeae* strains used in this study

Strain no.	Auxotype	Serovar	Fig./Lane
1	Pro ⁻	IB-1	1A
2	Pro ⁻	IB-2	1B
3	Pro ⁻	IB-3	1C, 3A
4	Pro ⁻	IB-4	1D
5	Pro ⁻	IB-5	1E
6*	Pro ⁻	IB-6	1F
7	Pro ⁻	IB-7	1G
8	Pro ⁻	IB-2	2A-2I
9	Pro ⁻	IB-3	3B
10	Proto	IB-3	3C
11	Proto	IB-3	3D
12	Proto	IB-3	3E
13*	Proto	IB-3	3F
14	Proto	IB-3	3G
15	Proto	IB-3	3H
16	Pro ⁻	IB-19	4A
17*	Pro ⁻	IB-19	4B
18	Pro ⁻	IB-19	4C
19*	Pro ⁻	IB-19	4D
20†	Proto	IB-19	4E
21	Proto	IB-19	4F
22	Pro ⁻	IB-7	—
23*	Pro ⁻	IB-2	—
24†	Proto	IB-4	—
25	Proto	IB-1	—
26	Pro ⁻	IB-4	—
27	Pro ⁻	IB-14	—
28	Pro ⁻	IB-6	—
29*	Pro ⁻	IB-6	—
30	Pro ⁻	IB-7	—
31	Proto	IB-7	—
32	Pro ⁻	IB-1	—
33	Pro ⁻	IB-7	—
34*	Pro ⁻	IB-7	—
35†	Pro ⁻	IB-6	—
36	Proto	IB-6	—
37	Pro ⁻	IB-7	—
38	Pro ⁻	IB-7	—
39	Pro ⁻	IB-2	—
40*	Pro ⁻	IB-9	—
41	Pro ⁻	IB-2	—
42	Pro ⁻	IA-8	—
43	Proto	IB-7	—
44	Pro ⁻	IB-6	—
45	Proto	IB-1	—
46	Pro ⁻	IB-1	—
47	Proto	IB-7	—
48†	Proto	IB-2	—

No superscripts indicate absence of plasmids.

Proto, prototrophic, wild-type.

Pro⁻, a requirement for proline.

* Presence of 7.2-kb plasmid.

† Presence of 7.2-kb and 39.2-kb plasmids.

1% at 42°C, distributed into the slots of a mold (BioRad Laboratories), and allowed to solidify. The agarose plugs were transferred into 10 ml of EC lysis solution (6 mM Tris-HCl, 100 mM EDTA, 1 M NaCl, brij-58 0.5%, sodium deoxycholate 0.2%, sodium lauroyl sarcosine 0.5%, 10 mg of lysozyme and 10 units of RNAase) and incubated overnight at 37°C with gentle shaking. After further incubation in ESP solution (0.5 M EDTA, pH 9–9.5; sodium lauroyl sarcosine 1%, proteinase K 500 µg/ml) for 2 days at 55°C with gentle shaking, the plugs were washed three times with 15 ml of TE buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA) for 30 min and stored at 4°C.

Restriction endonuclease DNA digestion

Prepared plugs were equilibrated in 10 mM Tris-HCl for 15 min on ice and digested overnight at 37°C in 150 µl of restriction buffer containing 10 U of endonuclease (New England Biolabs, USA).

Pulsed-field gel electrophoresis

Digested plugs were sealed into slots in an agarose (SeaKem GTG agarose, FMC Corp.) 1% gel and electrophoresed in a contour-clamped homogeneous electric field (CHEF) apparatus with a hexagonal electrode array (BioRad) for 20 h at 200 V with pulse times of 5–15 s. Gels were stained with ethidium bromide solution (1 µg/ml) for 60 min and then destained in distilled water for 30 min before being photographed under UV transillumination. *Rhodobacter sphaeroides* 2.4.1 genomic DNA digested by *AseI* was used as the mol. wt standard.¹⁴

Results

Selection of restriction endonuclease

Genomic DNA from *N. gonorrhoeae* strains was digested with the low-frequency cleavage (LFC) enzymes *NotI*, *SfiI* and *PacI* that recognise 8-bp sequences and *NheI*, *SpeI* and *XbaI* that recognise 6-bp sequences containing the tetranucleotide CTAG, an infrequent sequence in most bacterial genomes.¹⁵ *NotI* and *SfiI* failed to digest gonococcal DNA, presumably due to extensive DNA methylation. *PacI* produced suitable REA patterns but was too costly for routine use. *SpeI* and *NheI* produced suitable REA patterns, generating 12–17 and 14–16 fragments, respectively. Sufficient strain differences were observed for the development of a typing system based on restriction fragment length polymorphisms.

Typing of *N. gonorrhoeae* strains by *SpeI* and *NheI* digestion

Seven proline-requiring (Pro⁻) strains of differing serotype were examined. All showed distinct *SpeI* (fig. 1) and *NheI* REA patterns (data not shown).

The reproducibility of PFGE REA patterns generated by both *SpeI* and *NheI* was examined by analysis of the same bacterial strain on separate occasions. Identical patterns were observed for two repeat analyses (data not shown). Mobility of the DNA fragments can vary between analyses but the presence of standard DNA mol. wt markers on each gel enabled strict comparisons to be made between separate gels. Stability of the REA pattern was also observed when the same strain (strain 8, Pro⁻/IB-2) was subjected to nine in-vitro passages on MTM plates (fig. 2).

Comparison of PFGE analysis with A/S classification

Six strains of identical A/S (Prototrophic/IB-3) patterns were examined by PFGE analysis after *NheI*

Fig. 1. *SpeI* REA patterns of genomic DNA from *N. gonorrhoeae* Pro⁻ auxotrophs with different serovar designations. Lane A: strain 1 (IB-1); B: strain 2 (IB-2); C: strain 3 (IB-3); D: strain 4 (IB-4); E: strain 5 (IB-5); F: strain 6 (IB-6); G: strain 7 (IB-7).

Fig. 2. Stability of *SpeI* PFGE profiles of *N. gonorrhoeae* strain 8 (Pro⁻/IB-2) after serial passages (lanes A–I). Lane J: mol.wt marker (*R. sphaeroides* 2.4.1 digested with *AseI*).

digestion (fig. 3, lanes C–H). Two yielded identical patterns (lanes C and D), another was closely similar (lane E); the remaining isolates (lanes F, G and H) gave patterns distinct from each other and from this group. Two Pro⁻/IB-3 strains (lanes A and B) showed clearly distinct PFGE profiles. Thus, the eight IB-3 isolates were represented by two auxotypes but seven *NheI* PFGE REA patients. Of the six Prototrophic (Proto)IB-3 strains studied, two were from the same female patient and the PFGE profiles for these were

identical (lanes C and D). A closely related PFGE profile was found in strain 12 (lane E) which was isolated from a different patient. The three Proto/IB-3 strains exhibiting distinct PFGE profiles (lanes F, G and H) were collected from three separate individuals. Based on A/S classification, the five patients would appear to be infected with a common strain (Proto/IB-3) but subtyping by PFGE profiles showed that at least four strains were involved.

Similarly, six IB-19 strains of two auxotypes gave

Fig. 3. *NheI* digestion of DNA of *N. gonorrhoeae* Pro⁻/IB-3 and Proto/IB-3 strains. Lane A: strain 3 (Pro⁻/IB-3); B: strain 9 (Pro⁻/IB-3); C: strain 10 (Proto/IB-3); D: strain 11 (Proto/IB-3); E: strain 12 (Proto/IB-3); F: strain 13 (Proto/IB-3); G: strain 14 (Proto/IB-3); H: strain 15 (Proto/IB-3).

Fig. 4. *SpeI* digestion of DNA from *N. gonorrhoeae* Pro⁻/IB-19 and Proto/IB-19 strains. Lane A: strain 16 (Pro⁻/IB-19); B: strain 17 (Pro⁻/IB-19); C: strain 18 (Pro⁻/IB-19); D: strain 19 (Pro⁻/IB-19); E: strain 20 (Proto/IB-19); F: strain 21 (Proto/IB-19).

four *SpeI* PFGE profiles (fig. 4). With the exception of one strain (lane B), three Pro⁻/IB-19 strains (lanes A, C and D) displayed identical *SpeI* PFGE profiles. Two Proto/IB-19 strains were found to exhibit different *SpeI* PFGE profiles (lanes E and F). The identical

PFGE profiles observed in lanes A, C and D were generated by strains 16, 18 and 19, respectively, from three separate patients, and suggest a common source of infection.

Discussion

REA has been used extensively in the typing of diverse species of bacteria.¹⁶ Usually, this has relied upon electrophoresis patterns of the low-mol.-wt fragments produced by high-frequency cleavage (HFC) enzymes. Conventional agarose or polyacrylamide gel electrophoresis of low-mol.-wt fragments tend to generate complex REA patterns that are difficult to interpret. High-mol.-wt DNA fragments with no known cleavage sites for the particular restriction endonuclease chosen could not be resolved. Falk's study⁶ on REA pattern analysis relied mainly on *HindIII* digestion that generates fragments of < 5 kb in polyacrylamide gel electrophoresis. Agarose electrophoresis⁷ provided excellent resolution of fragments from 2 to 6.5 kb for *HinfI*-digested DNA and from 2.5 to 21.5 kb for *BglIII*-digested DNA. When REA patterns differed in these size ranges, their non-identity was clear, but strains sharing indistinguishable low-mol.-wt patterns might still differ in sequence in areas of the genome represented by the unresolved, high-mol.-wt DNA fragments in these digests. Falk *et al.*¹⁷ compared REA with A/S classification and found REA to be more discriminatory. Correlations were seen between some REA patterns and auxotypes.

However, they concluded that the choice and number of restriction endonucleases could influence the sensitivity of REA.

Fingerprinting by CHEF analysis provides a method for comparison of the whole genome including chromosome and plasmid components. However, resolution of plasmids or plasmid-derived fragments depends upon the presence of endonuclease target sequences and the sizes of the plasmids and fragments. Small plasmids and low-mol.-wt plasmid fragments can be resolved under certain PFGE conditions. When chromosomal PFGE patterns appeared identical, conditions could be chosen for resolution of small plasmids or plasmid-derived fragments. In this way, the whole genome of any strain could be screened. Another advantage is the cost saving on media and subculturing. In the method described here, growth from a single plate is used, whereas that of Falk *et al.*⁶ required growth from up to six plates. The cost of PFGE analysis is largely dependent upon the restriction endonucleases used. Although *SpeI* and *NheI* were equally discriminatory, the latter is preferred as it costs substantially less.

Reproducibility and stability of the REA patterns was confirmed by the identical fingerprints obtained from a single strain resolved in different gels and the stability of REA patterns over nine repeated subcultures. Recombination and chromosomal rearrangements at reiterated sequence¹⁸ sites might

change the restriction pattern but this was not observed during the present study.

The 48 clinical isolates studied were represented by two auxotypes and 11 different serovars. Combination of auxotyping and serotyping produced 18 A/S classes. The findings of 38 *SpeI* and 40 *NheI* CHEF REA patterns amongst the group of 48 isolates studied clearly demonstrated that PFGE was more discriminatory than either auxotyping or MAb-based serotyping. Strains were clearly differentiated and a positive correlation was observed in some strains between PFGE patterns and A/S patterns. Discrimination of strains within major serovars IB-1 and IB-3 was achieved. Serovar classes IB-5 and IB-7, which differ in their reaction with a single antibody, could also be differentiated and PFGE is clearly the method of choice for differentiation of strains that are A/S untypable. Clearly, a larger study, involving analysis of isolates from cohorts and covering a broad range of auxotypes and serovars is required, but the method shows considerable promise as a highly discriminatory tool for the epidemiological investigation of gonorrhoea.

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