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Genetic Diversity of *Neisseria gonorrhoeae* IB-2 and IB-6 Isolates Revealed by Whole-Cell Repetitive Element Sequence-Based PCR

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Phenotypic characterization of 19 *Neisseria gonorrhoeae* serovar IB-2 and 8 serovar IB-6 isolates by the combined use of auxotypes, serological characterization, and penicillin susceptibility testing indicated intraserovar genetic diversity. In the present study, we applied whole-cell repetitive element sequence-based PCR (rep-PCR) analysis which allows a rapid assessment of the clonal relationships of IB-2 and IB-6 isolates. DNA templates were prepared by boiling cells harvested directly from plate cultures, eliminating the need for time-consuming phenol extraction. Six different rep-PCR profiles were established among the 19 IB-2 isolates. Rep-PCR typing results had a good correlation with pulsed-field gel electrophoresis patterns. It is slightly less discriminatory than *Bgl*II-generated macrorestriction pattern analysis by pulsed-field gel electrophoresis. It is capable of discriminating epidemiologically related from epidemiologically unrelated IB-2 isolates. It should serve as a rapid and useful subtyping tool for epidemiologic investigations in which there is a predominance of major serovar groups.

Neisseria gonorrhoeae infections are among the most common sexually transmitted diseases worldwide. Progress in vaccine development is slow, but recent advances in understanding the immunomolecular biology of the gonococcus coupled with better access to experimental animal and human model systems could lead to a successful vaccine in the not too distant future. However, the mainstay in the prevention and management of gonococcal infections still rests on epidemiological studies, which have relied on the use of sensitive and highly discriminatory methods. These methods are necessary for monitoring the effectiveness of antibiotic treatment, contact tracing, screening, and identifying asymptomatic and high-risk individuals.

The auxotype-serovar (A/S) classification scheme is the most widely accepted method for the discrimination of gonococcal strains (8, 9). However, the A/S classification scheme has a number of limitations. These include the restricted supply of serotyping reagents, the batch-to-batch variations of monoclonal antibodies, and the reproducibility of coagglutination reactions, especially when using monoclonal antibodies such as 2D6, 2G2, and 6D9 (4). There is also skepticism that it may not provide sufficient discrimination of the strains present within major serovars such as IB-1 and IB-3 (9). Because there are problems with the reactivity of some strains with the 2D6 monoclonal antibody, classification of these into either the IB-2 or the IB-6 serovar could be ambiguous. In addition, the isolation of IB-2 and IB-6 serovar strains presenting with different auxotype and penicillin susceptibility markers indicates that the A/S scheme alone may be insufficient for subtyping strains.

Genotypic characterization of strains by pulsed-field gel electrophoresis (PFGE) of *Spe*I- and *Bgl*II-generated genomic restriction fragments was shown to be effective in subtyping *N. gonorrhoeae* serovar IB-2 and IB-6 strains (7). However, the high cost of the equipment for PFGE analysis coupled with the more technically demanding procedure may not be amenable to laboratories that already lack access to the A/S classification scheme.

In view of the practical limitations of PFGE, molecular subtyping methods that are rapid and simple to perform are needed to differentiate strains within the IB-2 and IB-6 serovars. Dasi et al. (1) had previously reported a low-stringency PCR amplification with arbitrarily chosen oligonucleotide primers to generate random amplified polymorphic DNA fingerprints. However, we found that the OPAA3 and OPAA13 primers (Operon Technologies, Alamanda, Calif.), as previously reported in those studies (1), could not be used to discriminate *N. gonorrhoeae* IB-2 and IB-6 serovar strains (unpublished data). In addition, the preparation of purified DNA templates from bacterial isolates can be time-consuming and costly.

In this report we describe the development of a rapid whole-cell-based repetitive element sequence-based PCR (rep-PCR) for the intraserovar discrimination of IB-2 and IB-6 serovar strains. In rep-PCR, consensus primers complementary to each end of a repeated sequence are used, and the differences in DNA band sizes observed after agarose gel electrophoresis reflect polymorphisms in the distances between repetitive sequence elements within the different genomes (5). Nineteen IB-2 and eight IB-6 isolates were characterized by the rep-PCR profiles developed in the present study, and these profiles were compared with those obtained by PFGE analysis of macrorestriction patterns.

MATERIALS AND METHODS

Bacterial strains. The 27 clinical isolates of *N. gonorrhoeae* used in the study and their respective A/S characteristics and penicillin susceptibility phenotypes are listed in Table 1. The strains comprised 10 selected WT/IB-2/S clones (wild-type strains susceptible to penicillin) identified as being epidemiologically related, 9 unrelated IB-2 isolates, and 8 randomly isolated IB-6 isolates. Strains were grown on modified Thayer-Martin agar (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.) and were incubated at 37°C in the presence of 5% CO_2 for 20 h.

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TABLE 1. Discrimination of *N. gonorrhoeae* IB-2 serovar strains by phenotypic characteristics, rep-PCR, and PFGE patterns

Strain no.	Auxotype[a]	Penicillin susceptibility[b]	Rep-PCR pattern[c]	PFGE pattern[d]	
				*Spe*I	*Bgl*II
92/049	WT	S	1	A	A
92/133	WT	S	1	A	Aa
92/213	WT	S	1	A	A
92/215	WT	S	1	A	A
92/336	WT	S	1	A	Aa
92/038	WT	S	1	A	Aa
92/239	WT	S	1	A	A
92/386	WT	S	1	A	A
92/413	WT	S	1a	A	A
92/320	WT	S	1b	A	A
91/238	WT	I	2	B	B
92/290	WT	I	3	C	C
92/411	WT	I	4	C	D
92/142	Pro	I	5	C	E
92/279	Pro	R	6	D	F
92/221	Pro	R	6	Da	F
92/053	Pro	I	1	A	A
91/302	Pro	S	1	A	Aa
91/421	Pro	S	1	A	Aa

[a] WT, wild type; Pro, proline requiring.
[b] R, resistant; I, intermediate; S, susceptible.
[c] The designations 1a and 1b denote clonal variants of the major rep-PCR type 1 clone.
[d] The designations Aa and Da denote clonal variants with a difference of three or fewer bands between electrophoretic patterns when compared with the number of bands in the major PFGE types.

Serological characterization. Strains were serotyped with monoclonal antibodies manufactured by Syva (Palo Alto, Calif.) according to the nomenclature of Knapp et al. (6) with six monoclonal antibodies specific for protein IB.

Antimicrobial susceptibility. The penicillin MICs for the isolates were determined by the agar dilution method (7). Isolates were classified as susceptible when the MIC was less than 0.03 mg/liter, intermediate when the MIC ranged from 0.06 to 0.5 mg/liter, and resistant when the MIC was found to be greater than 1 mg/liter.

DNA isolation. Two methods were used to prepare the DNA from *N. gonorrhoeae* for rep-PCRs.

(i) DNAs from whole cells were prepared by scraping three to four colonies (colony dimension, approximately 1 to 2 mm) from a Thayer-Martin agar plate and suspending the cells in 300 μl of 0.85% saline. Suspensions of cells were vortexed briefly and were centrifuged at 10,000 × *g* for 5 min. The supernatant was discarded and 300 μl of sterile double-distilled water was added. Cell suspensions were boiled for 15 min and were centrifuged at 12,000 × *g* for 2 min. The supernatant was removed, and 2 μl of each whole-cell preparation was used as the template DNA for PCR.

(ii) Purified DNA was obtained by suspending cells in 1 ml of 10× TNE (200 mM Tris-HCl [pH 8], 50 mM NaCl, 20 mM EDTA) containing freshly prepared lysozyme (1 mg/ml), and the mixture was left at room temperature for 1 h. Cells were lysed in TNE buffer containing 0.4% Triton X-100; this was followed by phenol-chloroform extraction. DNA was recovered by ethanol precipitation, redissolved in TE (10 mM Tris, 1 mM EDTA [pH 8.0]), and quantified spectrophotometrically.

Rep-PCRs. The repetitive element sequence-based primer sequences REPIR-Dt (5′-IIINCGNCGNCATCNGGC-3′) and REP-2-Dt (5′-NCGNCTT ATCNGGCCTAC-3′) (N = A, C, G, and T; I = inosine), which were described previously (3), were used for rep-PCRs.

PCRs. Reactions were performed in a volume of 25 μl containing 50 mM Tris-HCl (pH 9.0), 50 mM KCl, 7 mM $MgCl_2$, 200 μM (each) dCTP, dGTP, dATP, and dTTP (Pharmacia-LKB), 0.2 μM (each) primer, and 0.125 U of SuperTaq DNA polymerase (HT Biotechnology Ltd., Cambridge, United Kingdom). The amount of DNA varied according to the procedure that was used. For purified DNA, 100 ng of DNA was used, while 2 μl of each whole-cell preparation was used to provide template DNA for PCRs. Amplifications were performed in a DNA thermal cycler (480; Perkin-Elmer, Norwalk, Conn.) programmed as follows: initial denaturation of DNA templates at 95°C for 7 min and then 30 cycles comprising consecutive denaturation (30 s, 90°C), annealing (1 min, 45°C), and DNA chain extension (8 min, 65°C). A single final extension step at 65°C was carried out for 16 min. After PCR, 15-μl aliquots of the product were subjected to electrophoresis in 2% agarose gels; this was followed by ethidium bromide staining and photography under UV light.

PFGE. The PFGE method used in the present study has been described previously (7). Briefly, genomic DNAs entrapped in 1% agarose plugs were digested with *Spe*I or *Bgl*II and were electrophoresed in a contour-clamped homogeneous electric field (CHEF DRIII) apparatus with a hexagonal electrode array (Bio-Rad, Richmond, Calif.) at 14°C. The pulse time was ramped from 1 to 15 s for 8 h and then from 15 to 25 s for 16 h following *Spe*I digestion and from 1 to 15 s for 22 h following *Bgl*II digestion.

RESULTS

Phenotypic characterization of the 27 isolates by the combined use of auxotype, serological characterization, and penicillin susceptibility testing indicated the presence of subgroups within each of the two serovars. The complexity of performing the combined phenotypic tests could be circumvented by the rapid and simple whole-cell rep-PCR developed in the present study. When the rep-PCR fingerprints of each template DNA prepared from boiled cultures were compared with those obtained by standard phenol-chloroform extraction, there was no difference in the grouping of strains (data not shown). Six different rep-PCR profiles were established among the 19 IB-2 serovar strains examined by both approaches. Thirteen IB-2 serovar strains had the rep-PCR type 1 pattern (Fig. 1, lanes B to K and R to T), the type 2, 3, 4, and 5 patterns were each represented by a single isolate (Fig. 1, lanes L, M, N, and O, respectively), and the type 6 pattern was observed in two isolates (Fig. 1, lanes P and Q, respectively).

The eight IB-6 isolates could be allocated to six distinct rep-PCR groups on the basis of their overall rep-PCR patterns. Patterns 7, 8, 9, 11, and 12 were unique patterns exhibited by single isolates (Fig. 2, lanes B, C, D, H, and I, respectively), while three isolates generated indistinguishable fingerprints represented by pattern 10 (Fig. 2, lanes E, F, and G). The rep-PCR fingerprints of the eight IB-6 isolates were clearly different from those observed with the 19 IB-2 serovar strains.

The reproducibility of the rep-PCR patterns used for grouping the 19 IB-2 and 8 IB-6 isolates was tested three times with frozen cultures which had been kept at −20°C (over a period of 6 months). The rep-PCR fingerprints remained highly reproducible, except for differences in some band intensities (data not shown).

The rep-PCR typing results generally showed good agreement with the results obtained by PFGE analysis (Table 1). PFGE analysis with *Bgl*II-generated macrorestriction patterns

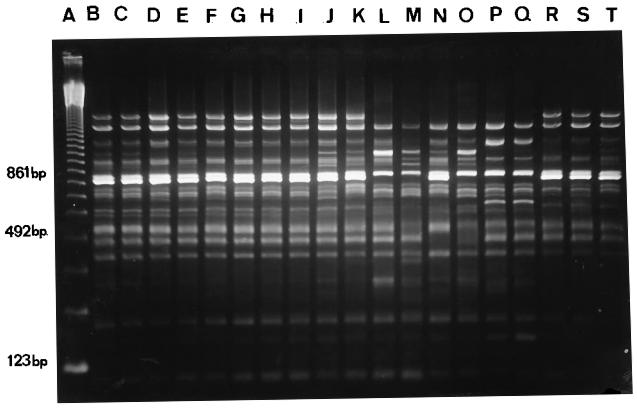

FIG. 1. Rep-PCR-based DNA fingerprints of 19 *N. gonorrhoeae* IB-2 isolates. Lanes B to T, isolates 92/049, 92/133, 92/213, 92/215, 92/336, 92/038, 92/239, 92/386, 92/413, 92/320, 91/238, 92/290, 92/411, 92/142, 92/279, 92/221, 92/053, 91/302, and 91/421, respectively; lane A, 123-bp DNA standard ladder.

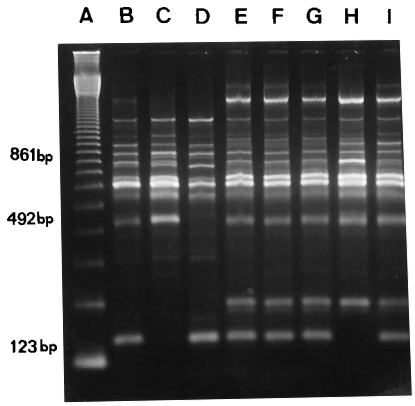

FIG. 2. Rep-PCR-based DNA fingerprints of eight *N. gonorrhoeae* IB-6 isolates. Lanes, B to I, isolates 90/125, 91/347, 92/208, 91/227, 91/236, 91/384, 91/275, and 91/378; lane A, 123-bp DNA standard ladder.

provided a slightly higher level of sensitivity than the results achievable by both *Spe*I restriction and rep-PCR analyses. For example, clonal variants with macrorestriction patterns (pattern Aa) highly related to that of the major clone represented by the type A pattern could be identified by *Bgl*II restriction and then PFGE analysis but not following *Spe*I restriction or rep-PCR analysis. PFGE analysis with *Spe*I also failed to differentiate three IB-2 isolates (strains 92/290, 92/411, and 92/142) which were subsequently resolved by using *Bgl*II macrorestriction patterns and rep-PCR profiling. On the other hand, two IB-2 proline-requiring (Pro/IB-2) isolates (strains 92/279 and 92/221) which were resistant to penicillin and which were found to have identical *Bgl*II macrorestriction patterns and rep-PCR profiles were shown to be clonal variants by *Spe*I restriction.

Both rep-PCR and PFGE analysis with *Spe*I failed to differentiate two isolates, namely, isolates 91/302 and 91/421, from an epidemiologically unrelated isolate, isolate 92/053. PFGE analysis with *Bgl*II was able to identify the two isolates as clonal variants of isolate 92/053. The higher discriminatory ability of PFGE analysis was also evident in the resolution of a group of three IB-6 isolates with identical rep-PCR patterns, pattern 10, into two isolates with the type H pattern and one isolate with the type I *Spe*I pattern. The same conclusion was reached by analysis of *Bgl*II-generated PFGE patterns. The heterogeneity of WT/IB-6 isolates was established by rep-PCR and PFGE analysis following *Bgl*II and *Spe*I restriction (Table 2).

DISCUSSION

In the present study, the use of the A/S classification system was found to be inadequate for the discrimination of both IB-2 and IB-6 strains isolated over a 1.5-year period from individuals in Sydney, Australia. An additional phenotypic marker such as susceptibility to penicillin was found to be useful for subtyping some WT/IB-2 and Pro/IB-2 strains. However, susceptibility or resistance to penicillin is a phenotypic trait, and such alterations in antimicrobial susceptibility without a genetic basis would not be effective in defining clonal relationships. For example, three WT/IB-2 isolates which exhibited intermediate susceptibility to penicillin would be classified as having a common clonal origin, but both rep-PCR and PFGE analysis established them to be of different clonal origins. Rep-PCR and PFGE analysis with *Spe*I identified three Pro/IB-2 isolates that had a clonal origin identical to that of the epidemic WT/IB-2/S (with the S indicating penicillin susceptibility) clone. The slightly higher sensitivity provided by analysis of *Bgl*II macrorestriction patterns identified two of the three isolates (Pro/IB-2/S) as clonal variants of the isolate (S92/053) which had intermediate susceptibility to penicillin. Both rep-PCR and PFGE analysis based on *Spe*I macrorestriction patterns indicated the existence of a common epidemic clone circulating in the community during 1991 and 1992. However, the higher sensitivity achieved by analysis of *Bgl*II macrorestriction patterns indicated the existence of the major epidemic clone WT/IB-2/S which was represented by the *Bgl*II type A pattern and its clonal variant represented by type Aa pattern. Three Pro/IB-2 strains which have clonal origins common with those of the epidemic IB-2 proline-nonrequiring (wild-type) strains could have arisen through mutations affecting only the proline biosynthetic pathway genes. These mutations do not cause extensive genome rearrangements or significant shifting of the DNA bands that can be detected by PFGE analysis. The mutations probably occurred in genomic regions lacking repetitive sequences because no polymorphisms were detected by rep-PCR.

Both the PFGE and rep-PCR profiles of the IB-6 strains were clearly different from those of the IB-2 strains. On the basis of phenotypic characteristics, two IB-6 isolates (90/125 and 91/347) which were wild type and which had intermediate susceptibility to penicillin would have been thought of as having a common clonal origin, but rep-PCR and PFGE indicated otherwise. Among the four WT/IB-6/S isolates, rep-PCR identified three to have a common clonal origin, and these three strains isolated in 1991 were clearly different from the clone circulating in 1992.

Molecular typing by whole-cell rep-PCR (14) has been found to be a useful technique for the epidemiological investigations of both gram-positive and gram-negative bacteria. It has been established to provide rapid assessment of clonal relationships for *Citrobacter diversus* (13) and *Streptococcus pneumoniae* (10). Unlike arbitrarily primed PCR (12), which requires the selection of appropriate primers, rep-PCR analysis readily allows examination of the entire genome with universal repetitive element sequence primers directed against sequences which are highly conserved across many different bacterial genera (11). We found the use of REPIR-Dt and REP-2-Dt primers to be very useful in the generation of highly discriminatory and reproducible rep-PCR profiles for *N. gonorrhoeae* serovars IB-2 and IB-6. The simple template preparation from boiled cells without the need for the time-consuming in vitro DNA extraction method involving phenol-

TABLE 2. Discrimination of *N. gonorrhoeae* IB-6 serovar strains by phenotypic characteristics, Rep-PCR, and PFGE patterns

Strain no.	Auxotype[a]	Penicillin susceptibility[b]	Rep-PCR pattern	PFGE pattern[c]	
				*Spe*I	*Bgl*II
90/125	WT	I	7	E	G
91/347	WT	I	8	F	H
92/208	WT	S	9	G	I
91/227	WT	S	10	H	J
91/236	WT	S	10	H	J
91/384	WT	S	10	I	Ja
91/275	WT	R	11	J	K
91/378	Pro	I	12	K	L

[a] WT, wild type; Pro, proline requiring.
[b] R, resistant; I, intermediate; S, susceptible.
[c] The designation Ja denotes a clonal variant with a difference of four bands between electrophoretic patterns when compared with the number of bands of the major PFGE type J pattern.

chloroform treatment coupled with 100% typeability should enable this approach to be easily adopted in most clinical microbiology laboratories.

We took several precautions to avoid the contamination problems that could be encountered in our PCRs. Specific workstations designated for PCR work were used, and sterile techniques were adopted. Specific pipettes and tips plugged with sterile cotton wool were used for each transfer of solutions. Small aliquots of reagents were used for the PCR, and the number of pipetting steps was reduced to a minimum. Negative control PCRs which did not include any DNA template were performed with the primers used in the present study. No DNA bands were observed in agarose gels following electrophoresis of the PCR mixtures.

We screened the rep-PCR patterns of every isolate to look for overall gross differences. The rep-PCR patterns of isolates assigned to different groups were significantly different. Because no universal guidelines are available for the interpretation of rep-PCR patterns, the comparisons of patterns remained subjective. We found that strains that were derived from the same outbreak had identical patterns and those that were closely related to the outbreak clone differed by only a few DNA bands. Strains that were epidemiologically unrelated showed rep-PCR patterns that were clearly different from that of the major outbreak clone. The rep-PCR approach appears to be well-suited for subtyping strains present within serovars IB-2 and IB-6, and it could be followed by PFGE analysis when higher resolution is warranted. However, despite its slightly higher discriminatory power, PFGE analysis is restricted to laboratories with the established technology because it is labor intensive and costly, and compared with rep-PCR analysis, it takes a longer period to yield results.

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