Persistence of Two Genotypes of *Neisseria gonorrhoeae* during Transmission

I. M. C. Martin,^{1*} A. Ghani,² G. Bell,³ G. Kinghorn,³ and C. A. Ison¹

Departments of Infectious Diseases and Microbiology¹ and Infectious Disease Epidemiology,² Faculty of Medicine, Imperial College London, London, and Department of Genitourinary Medicine, Royal Hallamshire Hospital, Sheffield,³ United Kingdom

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Isolates of *Neisseria gonorrhoeae* were tested using a highly discriminatory typing method, *opa* typing, to examine the genetic diversity over a 2-year study period of isolates from all consecutive patients with gonorrhea attending the Genitourinary Medicine clinic in Sheffield, United Kingdom. Two *opa* genotypes were detected throughout the 2-year time period and comprised 41% of all strains tested. The persistence of two *opa* types was investigated further to determine the apparent genetic stability, by examining the ability of isolates to undergo intragenic and intergenic recombination and mutation in vitro. Intragenic recombination or mutation involving the *opa* genes of *N. gonorrhoeae* in the selected isolates was not detected, but intergenic recombination did occur. *opa* genes of *N. gonorrhoeae* in vivo appear to diversify primarily through intergenic recombination. Intergenic recombination in vivo would require the presence of a mixed gonococcal infection, in which an individual is concurrently colonized with more than one strain of *N. gonorrhoeae*. We propose that the level of diversity of *opa* genotypes in a population is linked to the degree of sexual mixing of individuals and the incidence of mixed infections of *N. gonorrhoeae*.

Neisseria gonorrhoeae is a nonclonal bacterium that has a high rate of recombination and mutation, creating genetic diversity (6, 18, 19). The rate of genetic change of different variable genes through a transmission chain has not been determined, due to the difficulty in elucidating a well-defined transmission chain where all infected partners and their sexual history are known. However, it is widely thought that isolates of *N. gonorrhoeae* are concordant in phenotype and genotype across a short transmission chain, but not over a long period involving multiple individuals due to the extensive capacity for genetic variation (20). However, a short or long transmission chain has not been defined by the numbers of sexual partners. It has been shown that the majority of isolates of *N. gonorrhoeae* from sexual contacts have the same phenotype (3, 11) and genotype, determined by a variety of genotypic methods (10, 17, 22, 24, 25). As *N. gonorrhoeae* is highly efficient at all points of its life cycle for recombination (2), persistence of a particular genotype over time should not in theory occur, particularly in genes where genetic variations accumulate very rapidly.

In vivo during the course of infection with *N. gonorrhoeae* some of the organisms lyse, releasing free DNA that will be available for incorporation by recombination into the chromosome of other gonococci, contributing to the genetic variation. Intergenic recombination, recombination between different gonococci, might occur during mixed infections of *N. gonorrhoeae*, in which an individual is infected with more than one strain in the same anatomical site (3, 7, 9, 20, 21). Evidence for mixed gonococcal infections—which are expected to occur in

highly sexually active individuals, generating new genotypic strains—has recently been demonstrated (15). Recombination between gonococci of the same genotype, intragenic recombination, has been shown to occur in vivo and in vitro (1, 6, 26), resulting in greater genetic diversity. By using highly discriminatory typing techniques, it is possible to monitor the transmission and genetic variation of strains of *N. gonorrhoeae* between individuals, discriminating between linked and nonlinked individuals. *opa* typing (20), which indexes the variation within the 11 gonococcal *opa* genes that encode outer membrane proteins, involved in adherence and invasion of epithelial cells, has been shown to be able to confirm sexual contacts (10, 24, 25) and is currently one of the most discriminatory genotypic method to differentiate between gonococcal strains (23).

This study aimed to monitor strain variation and transmission of gonorrhea within a city with a single clinic, the Genitourinary Medicine (GUM) clinic, over a 2-year period, by collecting isolates of *N. gonorrhoeae* from consecutive patients with gonorrhea and characterizing the strains by *opa* typing. The underlying mechanism of persistence of genotypes was also investigated.

MATERIALS AND METHODS

Collection and characterization of isolates. Isolates of *N. gonorrhoeae* were obtained from all infected patients attending the GUM clinic at the Royal Hallamshire Hospital, Sheffield, United Kingdom, from April 1995 to March 1997. Isolates from the same individual were included only when more than 28 days had elapsed between the positive cultures for their first and repeat infections, to exclude isolates that were potential treatment failures. Test of cure in the United Kingdom is recommended within 1 month of treatment for gonorrhea (<http://www.agum.org.uk/guidelines.htm>). *N. gonorrhoeae* was isolated on Columbia agar base medium (Unipath, Basingstoke, Hampshire, United Kingdom), supplemented with 5% lysed horse blood, vancomycin (3 mg/liter), trimethoprim (5 mg/liter), and colistin (8 mg/liter). Isolates were saved in 15% glycerol broth and stored at –80°C. Isolates were retrieved as previously described (25). All strains were typed on the basis of their nutritional requirement, by testing for the ability to utilize proline, ornithine, hypoxanthine, methionine, uracil, and histidine (4). Serotyping was performed using the method and nomenclature of

* Corresponding author. Mailing address: Iona Martin, Department of Infectious Diseases and Microbiology, Faculty of Medicine, Imperial College London, St. Mary's Campus, Norfolk Place, London W2 1PG, United Kingdom. Phone: 44 207 594 3957. Fax: 44 207 262 6299. E-mail: i.martin@imperial.ac.uk.

Knapp et al. (12). *opa* typing of all isolates was performed using the method of O'Rourke et al. (20) as previously described (25). Briefly, *opa* genes were amplified by PCR from a bacterial lysate, which was then purified by gel excision using a Nucleiclean kit (Sigma, Poole, Dorset, United Kingdom). The product was then digested with the restriction enzyme *TaqI* using the recommended method of the manufacturer (New England Biolabs, Hitchin, Hertfordshire, United Kingdom). The resulting fragments were end labeled with [³²P]dCTP (ICN, Basingstoke, Hampshire, United Kingdom) and separated by electrophoresis on a 6% nondenaturing polyacrylamide gel. Profiles were analyzed using Gelcompar (Applied Maths, Kortrijk, Belgium) and were considered indistinguishable only if all bands were identical.

Patient data. Contact tracing data from patients with gonorrhea were obtained by interviews with health advisers at the GUM clinic as previously described (25). Data including age, ethnicity, partial postal code, and information on sexual partnerships in the previous 3 months were sought. Ethics approval was obtained. Patients were offered the choice of notifying their own sexual partners of their infection or providing contact information in order for the health adviser to inform the sexual partners confidentially.

Intragenic recombination. Four isolates from each of the two *opa* type clusters (*opa-1* and *opa-4*), two from women and two from men, one from each year, were subcultured daily using a sweep from multiple colonies on GC agar (Difco, Becton Dickinson, Oxford, United Kingdom) supplemented with 1% IsoVital X (BBL, Becton Dickinson, Oxford, United Kingdom). DNA lysates were prepared on days 1, 7, 14, 21, and 28 and used as the DNA source for *opa* typing (25).

Intergenic recombination. Transformation experiments were performed using chromosomal DNA from donor strains FA19 Rif^r (rifampin-resistant gonococcal strain FA19) (13) or ciprofloxacin-resistant strains that had a single *gyrA* mutation (*gyrA* AA91 = Phe), or double *gyrA* mutation (*gyrA* AA91 = Phe, AA95 = Gly). DNA was extracted according to the manufacturer's recommended method using a Qiaamp kit (QIAGEN, Crawley, West Sussex, United Kingdom), and the concentration of the chromosomal DNA was determined using a spectrophotometer (Shimadzu, Milton Keynes, Buckinghamshire, United Kingdom) with its DNA concentration program. The absorbance of the sample was measured at 260 and 280 nm; a 50-μg/ml concentration of double-stranded DNA gives an optical density (OD) of 1.0 at 260 nm, and the ratio of the OD readings provides an estimate of the purity of the DNA.

Transformation experiments were performed using a suspension of a pilated culture of the recipient isolate prepared in Proteose Peptone with an OD at 540 nm of 1.0 (11). Recipient isolates for the transformation were two isolates from each of the two *opa* type clusters, the *opa-1* and *opa-4* clusters, and a clinical isolate that had not been stored at -80°C; all of the isolates were sensitive to rifampin and ciprofloxacin. One hundred microliters of the recipient suspension was placed onto a GC agar plate to which 100 μl of 0.5-μg/ml chromosomal DNA from FA19 Rif^r was added, and the plate was incubated at 36°C with 5% CO₂ for 6 h. The growth was suspended in 1 ml of Proteose Peptone (Difco, Becton Dickinson), 100 μl was placed onto a GC agar plate containing rifampin (50 μg/ml) in duplicate, the plate was incubated for up to 5 days at 36°C with 5% CO₂, and the number of transformants was determined. The total viable count was determined on GC agar. The experiments were repeated using the same recipients with donor DNA from the ciprofloxacin-resistant isolates. Selection of transformants was performed on GC agar plates containing ciprofloxacin at a concentration of 1.0 μg/ml for the DNA from the donor isolate with the double *gyrA* mutation (MIC of ciprofloxacin, 16.0 μg/ml) or 0.12 μg/ml for the DNA from the donor isolate with a single *gyrA* mutation (MIC of ciprofloxacin, 1.0 μg/ml). Negative controls to which no donor DNA was added were included in each experiment. Transformation experiments were repeated three times. A selection of transformants from each experiment were autotyped, serotyped, and *opa* typed.

por gene sequencing. Eight isolates from each of the two *opa* type clusters that were isolated throughout the 2-year period were selected. Four control isolates with the same autotype and serovar as the two clusters, isolated from patients attending the GUM clinic at St. Mary's Hospital, London, United Kingdom, were also sequenced with respect to the *por* gene. The *por* gene was amplified from DNA lysates, using primers spanning from the noncoding region, based on the numbering of FA19 (14) (por1, 5'-⁷²GAATTCAAGCTTATGAAAAATCCC TGATTGCC¹⁰⁵-3') to the postcoding region (por2, 5'-¹⁰⁵²GTGACCTGC AGTTAGAATTTGTGGCGCAGA¹⁰⁶⁴-3'). Each PCR contained a 200 μM concentration of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP) (Roche, Lewes, East Sussex, United Kingdom), 10 μl of PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl) (Gibco BRL, Paisley, United Kingdom), 1.5 mM MgCl₂, 50 pmol of each primer, and 5 μl of DNA lysate. The reaction mix was overlaid with a drop of mineral oil, the samples were heated to 95°C using a Hybaid Ominigene PCR machine for 5 min, and then 2.5 U of *Taq* DNA

TABLE 1. *opa* clusters in isolates of *N. gonorrhoeae* from Sheffield, defined by number and size

Cluster size	No. of separate clusters (no. of isolates)
1.....	75 (75)
2.....	21 (42)
3.....	11 (33)
4.....	1 (4)
5.....	2 (10)
6.....	2 (12)
7.....	1 (7)
62.....	1 (62)
69.....	1 (69)
Total.....	115 (314)

polymerase (Gibco BRL) was added. Amplification was performed using 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 5 min, and then the mixture was held at 4°C.

The PCR product was purified by gel excision using a GeneClean kit (Bio 101, Stratagene, Amsterdam, The Netherlands). The sequencing reaction was performed using six internal primers (por3, 5'-⁴⁹⁵TTCAGGCTACCGGCGGATG ⁴⁷⁵-3'; por4, 5'-⁶³⁸TTCAGCGGCAGCGTACAATAC⁶⁵⁸-3'; por5, 5'-⁸⁸³GACG TACAGGGCATTATTGTC⁸⁶³-3'; BDR 81, 5'-⁴⁷⁶ATCCGCGCCGGTAGCCT GAAC⁴⁹⁶-3'; BDR 82, 5'-⁶⁵⁸GTATTGTACGCTGCCGCTGAA⁶³⁷-3'; and BDR 83, 5'-⁸⁶⁰TACGACAATAATGCCCTGTAC⁸⁸⁰-3'). Each reaction mix contained 8 μl of terminator ready reaction mix (Perkin-Elmer, Cambridge, United Kingdom), 6 μl of purified PCR product, 3 pmol of primer, and water to a final volume of 20 μl. Amplification was performed on a Robocycler (Perkin-Elmer) at 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min for 25 cycles, and then the mixture was cooled to 4°C. The sequencing reaction product was purified by ethanol precipitation. To each 20-μl volume, 2 μl of 3 M sodium acetate, pH 4.6, and 50 μl of 95% ethanol was added; the solution was then vortexed and placed on ice for 10 min. The solution was then centrifuged at 5,000 × g for 20 min before the supernatant was removed. The pellet was washed in 250 μl of 70% ethanol and centrifuged for 10 min at 5,000 × g, and the supernatant was removed. The pellet was dried on a hot block, prior to the addition of 25 μl of template suppression reagent (Perkin-Elmer). The sample was vortexed and centrifuged to form a pellet and was heated to 95°C for 2 min to denature. The sample was vortexed and centrifuged to form a pellet again and was loaded onto an ABI Prism 310 sequencer (Perkin-Elmer). Sequences were analyzed and aligned using Sequencher (Gene Codes Corporation, Ann Arbor, Mich.).

Statistical analysis. Chi-square tests, where appropriate, were performed in Microsoft Excel.

Nucleotide sequence accession numbers. All sequences have been submitted to GenBank and have the following accession numbers: AY297696 to AY297711.

RESULTS

During the 2-year study period, April 1995 to March 1997, 314 isolates of *N. gonorrhoeae* were collected. One isolate per patient per visit was included. The 314 isolates were from 300 individuals (143 men and 157 women), 14 of whom (8 women and 6 men) had repeat infections. Information about sexual partners from contact tracing was available on 267 index cases (85.0% overlap of the data sets).

opa typing. *opa* typing revealed 115 different *opa* profiles (Table 1) within the 314 isolates, of which 75 (23.9%) were unique, and two different types, previously designated the *opa-1* and *opa-4* clusters (25), accounted for 41.7% of the strains. The two large clusters of 62 (*opa-1*) and 69 (*opa-4*) isolates with indistinguishable *opa* profiles persisted throughout the 2-year study period (Fig. 1).

The *opa-1* cluster was composed of 62 isolates, 57 of which were of a PA(O)U IB-2 phenotype and 5 of which had a different serovar (IB-1, IB-16, and IB-26) that occurred spo-

A

B

FIG. 1. (A) Total number of isolates of *N. gonorrhoeae* collected by month and the incidence of *opa-1* and *opa-4* isolates; (B) distribution of *opa-1* and *opa-4* clusters in relation to the proportion of isolates collected by month.

TABLE 2. Demographic data arranged by cluster

Parameter	<i>opa-1</i> cluster (<i>n</i> = 49)		<i>opa-4</i> cluster (<i>n</i> = 63)		All other clusters and unique <i>opa</i> types	
	Male (<i>n</i> = 17)	Female (<i>n</i> = 32)	Male (<i>n</i> = 26)	Female (<i>n</i> = 37)	Male (<i>n</i> = 94)	Female (<i>n</i> = 61)
Ethnic group (no.)						
White	8	25	6	34	53	52
Black	9	6	19	1	18	6
Other	0	1	1	2	3	3
Postal code						
Central Sheffield	13	24	20	34	52	46
Outer Sheffield	4	6	6	3	42	15
Age (yr)						
Mean (SD)	25.4 (5.3)	19.6 (3.5)	25.3 (7.7)	23.1 (5.7)	27.1 (7.1)	23.9 (7.1)
Range (yr)	15–36	15–28	17–49	16–36	15–45	15–47
Median no. of reported contacts (range)	2 (0–8)	1 (0–3)	2 (0–4)	1 (0–34)	1 (1–26)	1 (1–22)
No. of screened contacts/no. of unscreened contacts	19/27	26/14	26/18	37/71	86/114	66/117

radically throughout the time span. Information about sexual partners was available for 49 of the 62 patient episodes. There were some known transmission links within the cluster: three pairs, three triplets, one group of five, and 10 links to individuals who did not have an *opa-1* profile, with the remaining individuals for whom isolates were available not reporting any direct sexual contacts. The median number of contacts, traced and untraced, for women was 1.0 (range, 0 to 3; mean, 1.25), and that for men was 2.0 (range, 0 to 8; mean, 2.7). (Table 2). All of the individuals were heterosexual, and there were significantly more women within the *opa-1* cluster than in other clusters ($P = 0.003$ [χ^2 test]).

The *opa-4* cluster was composed of 69 isolates, of which 66 isolates had an A IB-3 phenotype and 3 had a different phenotype (A IA-2 and NR IB-3) that occurred in essentially three separate periods throughout the 2 years—April to June 1995, October 1995 to January 1996, and May to August 1996—with no more instances of this *opa* type being isolated after November 1996. Information about sexual partners was available for 63 of the 69 patient episodes. The known transmission links within the cluster consisted of six pairs, three triplets, and 13 links to individuals with different *opa* types. The median number of contacts, traced and untraced, for women was 1.0

(range, 0 to 34; mean, 2.9), and that for men was 2.0 (range, 0 to 4; mean, 1.7) (Table 2). All individuals were heterosexual, and again there were significantly more women within the *opa-4* cluster than in other clusters ($P = 0.01$ [χ^2 test]). The men within the *opa-4* cluster were significantly more likely to be of a black ethnic group than were men within other clusters ($P = 0.04$ [χ^2 test]). The majority of individuals within the cluster had central Sheffield postcodes, which was significant compared to individuals not within the large clusters ($P = 0.01$ [χ^2 test]) (Table 2).

Recombination. The *opa* profiles of the isolates chosen from the clusters that were subcultured in vitro for 4 weeks were indistinguishable at each time point. Intragenic recombination or mutation affecting the *opa* genes of the isolates was not detected.

All isolates tested were transformable and produced rifampin-resistant colonies. Isolates of the *opa-4* cluster produced a significantly higher mean number of rifampin-resistant transformants per microgram of donor DNA ($P < 0.001$) than isolates of the *opa-1* cluster (Table 3). No spontaneous rifampin-resistant mutants occurred. Transformation using ciprofloxacin-resistant DNA as the donor again produced transformants with isolates from both clusters, with a higher mean

TABLE 3. Transformation frequencies using rifampin- and ciprofloxacin-resistant (single *gyrA* mutation) donors^a

Strain	<i>opa</i> type	Phenotype	Rifampin				Ciprofloxacin			
			Donor		Transformants		Donor		Transformants	
			Mean no. of transformants/ μ g of donor DNA (SD)	Mean frequency of transformants (SD)	<i>opa</i> type	Phenotype	Mean no. of transformants/ μ g of donor DNA (SD)	Mean frequency of transformants (SD)	<i>opa</i> type	Phenotype
<i>opa-1</i> strain	1	PAOU IB-2	12.62 (5.82)	1.37×10^{-7} (2.17×10^{-6})	1	PAOU IB-2	0.25 (0.16)	2.85×10^{-7} (4.03×10^{-7})	1	PAOU IB-2
<i>opa-4</i> strain	4	A IB-3	26.40 (2.32)	1.03×10^{-3} (2.4×10^{-3})	4	A IB-3	10.32 (6.02)	2.89×10^{-4} (3.94×10^{-4})	4	A IB-3
Clinical isolate		NR IB-6	16.45 (5.01)	2.12×10^{-5} (8.9×10^{-6})		NR IB-6	15.22 (12.42)	2.10×10^{-4} (2.54×10^{-4})		NR IB-6

^a Values are means of results of three experiments for ciprofloxacin transformation and four experiments for rifampin transformation. Values for the *opa-1* and *opa-4* groups are means for two separate isolates from the clusters. Twenty rifampin- and 20 ciprofloxacin-resistant transformants of each *opa* group (10 per strain) from each of the separate transformation experiments were *opa* typed and phenotyped.

number of transformants when DNA with a single *gyrA* mutation was used than when DNA with a double *gyrA* mutation was used. The 80 selected ciprofloxacin- and rifampin-resistant transformants (10 ciprofloxacin and rifampin transformants of each of the two cluster isolates from *opa-1* and *opa-4*) that were auxotyped, serotyped, and *opa* typed showed no variation between transformants from the same experiment. The phenotypes of the transformants were the same as those of the original isolate in both auxotype and serovar. The *opa* profiles of the transformants were the same as those of the original isolate, either *opa-1* or *opa-4*.

***por* sequencing.** The *por* gene nucleotide sequences of the isolates with an *opa-1* profile from throughout the 2-year period were identical, with no base changes (GenBank accession numbers AY297696 to AY297711). The amino acid sequences of the isolates with an *opa-4* profile were also identical to each other, but two isolates had separate single-nucleotide changes that did not alter the encoded amino acid sequence. The *opa-4* and *opa-1* sequences were different from each other. The four control isolates with the same phenotype as *opa-1* and *opa-4* isolates (PAOU IB-2 and A IB-3, respectively) each had different *por* sequences from each other and from the *opa* type clusters.

DISCUSSION

Through *opa* typing of isolates of *N. gonorrhoeae*, clusters of isolates with the same *opa* profile that have spanned a 2-year period in Sheffield were identified. The two clusters were shown to represent endemic transmission of gonorrhea in Sheffield over a 1-year period (25), and it appears that this has continued. The contact tracing revealed transmission links between infected individuals, but these could not fully explain the persistence of the two clusters. Patients are sometimes unwilling or unable to fully reveal all their sexual contacts. There were also numerous untraced contacts within each of the clusters.

N. gonorrhoeae is a nonclonal bacterium that exhibits a high level of genetic variation through recombination and mutation of surface exposed proteins, preventing the establishment of a protective immune response (13, 27). Not all gonococci have the same rate of genetic variation with more clonal lineages, such as AHU IA-2 strains, which diversify more slowly than other phenotypic strains (8). PAOU IB-2 and A IB-3 strains, the *opa-1* and *opa-4* phenotypes, are not known to be of a more clonal lineage; therefore, the persistence of the two genotypes was surprising. The persistence may have been due either to specific properties of the gonococci or to the fact that the individuals within the clusters had different sexual mixing patterns.

Intragenic recombination and mutation were not detectable in the *opa* genes of the selected isolates from either of the two clusters over the 4 weeks of in vitro subculture. It is possible that in vitro recombination did not occur and not that the gonococcal strains were incapable of intragenic recombination. In vivo it is possible that intragenic recombination and mutation are more frequent, but the relationship between the stability of *opa* genes in vivo and in vitro is not clear. There were some sexual contacts who had dates of attendance at the GUM clinic more than a month apart who were concordant in *opa*

type and phenotype, perhaps indicating that in vivo variation of *opa* genes has not occurred over a long time, supporting the in vitro findings. Intergenic recombination of gonococci with an *opa-1* or *opa-4* genotype was detected, and their stability was not due to an inability to recombine. In vivo intergenic recombination between different gonococci may occur during a mixed infection (15), and the resulting incorporation of DNA into the chromosome is likely to change the genotype of the strain, depending on which gene(s) was affected by the exchange of DNA. The factors influencing which genes undergo recombination in vitro or in vivo are not known, but typically up to a few kilobases of DNA are replaced (16). The finding that the selected isolates within the two separate clusters had the same *por* sequence supports the *opa* typing results, which has been previously shown (10, 24) and which suggests circulation of a single strain within each cluster.

From these results we hypothesize that in vivo variation of *opa* genes and the resulting *opa* profile occur predominantly through intergenic recombination, rather than intragenic recombination and mutation. Therefore, the diversity of *opa* types is dependent on the number of different strains of *N. gonorrhoeae* recombining in vivo during mixed infections. If no mixed infections occur in vivo the diversification of *opa* genes is likely to be reduced. The risk of a mixed infection occurring in an individual depends on the sexual activity of that person, the person's position within a sexual network, and also the sexual activities of the person's sexual partners. Within a local population, mixed gonococcal infections within the same individual are more likely in those with higher rates of partner change when there are a variety of different circulating strains. Therefore, in highly sexually active populations a greater level of diversity of *opa* genes and *opa* types is expected to occur (23, 25). However, if there were a limited number of circulating strains, or a single strain within a highly sexually active group, the potential for *opa* gene variation, or that of any other surface exposed protein, would be reduced by the reduction in potential for a mixed gonococcal infection.

Within the clusters in Sheffield where the two clusters were supported over a long period, it appears that there was assortative or restricted sexual mixing, which reduced the likelihood of mixed infections occurring and therefore maintained the genotype. The sexual activities of individuals within the clusters must have been at a level where on average one sexual contact became infected with *N. gonorrhoeae* to allow the persistence of the two *opa* type strains. It is also conceivable that in vivo the cluster isolate strains were inhibited in some way from undergoing intergenic recombination on contact with DNA from gonococci of another genotype. This may be possible due to strain dominance, which has been suggested as a possible reason for the clonal nature of AHU IA-2 strains (8).

Investigation of *opa* gene variation in vitro and in relation to epidemiologically identified transmission links appears to indicate that variation in vivo occurs more frequently through intergenic recombination. In vivo intergenic recombination would occur during mixed gonococcal infections, which is related to the sexual activities of individuals and the prevalence of gonorrhea. Hence, the degree of genetic diversity exhibited by *N. gonorrhoeae* is a reflection of the level of sexual activity.

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