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Typing of *Neisseria gonorrhoeae* Reveals Rapid Reinfection in Rural South Africa

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A recent study afforded us the opportunity to collect pre- and post-treatment isolates of *Neisseria gonorrhoeae* from women who supposedly failed to eradicate the organism when tested 8 to 10 days following treatment with a single, directly observed 250-mg dose of ciprofloxacin. In an attempt to differentiate true treatment failure from reinfection, we determined the ciprofloxacin MICs and performed auxotyping, serotyping, and opa typing of the pre- and post-treatment isolates. Paired isolates of *N. gonorrhoeae* were obtained from seven different women, despite susceptibility of the initial isolates to ciprofloxacin. Six of seven patients were infected with gonococcal isolates that differed significantly from their primary isolate. These most probably represent reinfection with a different strain, which could originate from the same partner infected with multiple strains or reinfected with a new strain or from a different partner. The susceptibility to ciprofloxacin of all isolates makes the possibility of multiple strains in the patient unlikely. The diversity of the isolates within the pairs therefore suggests rapid reinfection within the partnerships.

Infections with *Neisseria gonorrhoeae* are of concern to health care providers because they carry an increased risk of morbidity and mortality among women (3) as well as neonates (6). In addition, as with the other causes of sexually transmitted infections (STIs), they represent an elevated risk for infection with human immunodeficiency virus (HIV) (2, 5). Epidemiological surveillance including accurate strain identification is pivotal to the understanding of the transmission dynamics of this organism, which in turn influences public health interventions.

In a recent study, we evaluated the efficacy of the drugs employed in the syndromic management of nonulcerative STIs among women (8). Twenty percent of women treated with ciprofloxacin for culture-proven infection with *N. gonorrhoeae* remained culture positive when tested between 8 and 10 days later. We attributed the subsequent infection to either lack of compliance with antibiotic therapy, failure of the ingested antibiotics to eradicate the infecting organism, or reinfection.

In our setting, ciprofloxacin is the drug of choice for the treatment of cervicitis due to *N. gonorrhoeae* and is administered as a directly observed single dose. Lack of compliance therefore cannot explain the subsequent infection. Although the MICs of ciprofloxacin for *N. gonorrhoeae* have started to increase in this area (7), clinical failures have not as yet been seen. Attempts at partner notification and treatment have been largely ineffectual (16). We suspect that reinfection is common, but this has not been proven.

In an attempt to differentiate true treatment failure from reinfection, we determined the phenotype by using auxotyping, serotyping, and antibiograms of pre- and post-treatment isolates and examined the genetic relatedness by means of opa typing. Our hypothesis was that the isolates within each pair would be susceptible to ciprofloxacin, display identical antibiograms, and have identical phenotype and genotype. We expected these pairs to be identical based on the assumption that reinfection would be from the same partner, who would harbor a single strain of *N. gonorrhoeae.*

MATERIALS AND METHODS

Patients and isolates. The Africa Centre for Health and Population Studies primary-care clinic is situated in KwaMsane, a large periurban, combined formal and informal settlement on a major highway in the Hlabisa subdistrict of northern KwaZuluNatal.

We tested the efficacy of syndromic drug therapy in women with nonulcerative genital disease who attended this clinic between March 1999 and February 2000 (8). In this study, 692 women were treated for vaginal discharge by using the syndromic approach. Eighty-six (12%) were infected with *N. gonorrhoeae* at baseline. Of the 290 patients returning for follow-up, 51 had positive cultures for *N. gonorrhoeae* at baseline, of whom 10 (20%) had positive cultures at follow-up 8 to 10 days later. In 3 of these 10 pairs, one or both isolates could not be recovered from the freezer. Therefore, only seven pairs were available for typing.

Single colonies of *N. gonorrhoeae* isolates were subcultured and stored as suspension in glycerol-peptone at −70°C. MIC determination and typing were performed on organisms grown from these suspensions.

Typing. Auxotyping was performed using the method described by Copley and Egglestone (1). For serotyping the standard set of monoclonal antibodies and nomenclature described by Knapp et al. was used (4). MIC determinations were performed on all isolates for penicillin, tetracycline, spectinomycin, ceftriaxone, ciprofloxacin, and ofloxacin by using the National Committee for Clinical Laboratory Standards (NCCLS) defined method (9). In brief, twofold serial dilutions of antibiotics were added to molten GC agar base (Oxoid Ltd.) plus 1% Iso VitaleX supplement at 45°C. After solidification, these plates were seeded with 10^4 CFU of *N. gonorrhoeae* per spot by means of a multipoint inoculator. The plates were incubated at 37°C under CO_2 for 24 h. *N. gonorrhoeae* ATCC 49226, *Staphylococcus aureus* ATCC 25923, and *Escherichia coli* ATCC 10418 were used as controls. Isolates were tested for β-lactamase production by the chromogenic cephalosporin method. PCR detection and characterization of the *tetM* gene were performed on isolates of *N. gonorrhoeae* by using a one-step PCR amplification (13). PCR products were electrophoresed in a 1% agarose gel containing ethidium bromide and visualized by UV fluorescence.

Opa typing of the isolates was performed using the method of O'Rourke et al.

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TABLE 1. Sexual history of seven women culture positive for *N. gonorrhoeae* 10 days after ciprofloxacin treatment

Patient	Age (yr)	Age at coitarche (yr)	No. of lifetime sexual partners	RF[a]	No. of partners in last month	No. of current partners	Location of current partners(s)	Condom use
A	30	15	4	0.25	1	1	Local, separate	Never
B	23	17	3	0.5	1	1	Local, together	Never
C	21	20	7	7	1	1	Local, separate	Never
D	20	15	2	0.4	2	2	(i) 240 km away; (ii) local, separate	Occasional
E	17	16	2	2	1	1	250 km away	Occasional
F	21	17	3	0.75	1	1	Local, separate	Never
G	22	16	3	0.5	1	1	50 km away	Never

[a] RF = number of lifetime sexual partners/(age − age at coitarche).

(10). Briefly, the *opa* genes were amplified from gonococcal chromosomal DNA by PCR using a single pair of primers. The products were digested with frequently cutting restriction enzymes (*Taq*I and *Hin*PI). The restriction fragments were end labeled with ^{32}P and separated on polyacrylamide gels, and band patterns were compared.

RESULTS

Paired isolates of *N. gonorrhoeae* were obtained from seven different women. The demographic data for these patients are shown in Table 1. Their ages ranged from 17 to 30 years, and their risk factor (RF) for acquiring an STI (defined as number of partners divided by present age minus age at coitarche) ranged from 0.25 to 7 (median 0.5).

Table 2 shows the susceptibility of the *N. gonorrhoeae* strains to the antibiotics. Of the 14 isolates, 2 produced β-lactamase and both were isolated from the same patient (patient D). Only three isolates did not harbor the *tetM* gene. Two formed the pair isolated from patient E, and the third was the subsequent isolate from patient A. Her initial isolate did not carry this gene. Isolate B2 showed an increase in fluoroquinolone MIC compared to B1, exhibiting reduced susceptibility to ciprofloxacin and resistance to ofloxacin. The MICs of spectinomycin and ceftriaxone for the 14 isolates did not differ.

The results of auxotyping and serotyping are shown in Table 3. Of the 14 isolates, 10 were of the NR auxotype, 1 (B2) had the ARG auxotype, and 3 (A2, E1, and E2) had the PRO auxotype. Serotypes differed considerably, with IB3 being the dominant type (five isolates). Only two pairs showed identical serotypes: D1 and D2 both belonged to the A-NT type, while E1 and E2 belonged to IB3.

The *Taq*I and *Hin*PI opa types of the paired isolates are shown in Fig. 1. Of the 14 isolates, 8 produced unique opa types with both enzymes. One pair (D1 and D2) was indistinguishable by opa typing with both restriction enzymes. Two other isolates were indistinguishable but belonged to two different individuals (isolates C2 and F1). A further two isolates (B2 and F2) were indistinguishable by *Taq*I but produced different band patterns by *Hin*PI and belonged to different individuals.

Of the four typing methods, opa typing was most discriminative, followed by serotyping. Auxotyping and antibiogram typing did not have much discriminative power.

Isolate A1 differed from A2 in its susceptibility to tetracycline. Isolate A1 appeared more resistant than isolate A2 and harboured the *tetM* gene. These isolates belonged to different auxotype/serotype (A/S) classes as well as to different opa

TABLE 2. Drug MIC, presence of β-lactamase, and *tetM* genes of pre- and post-treatment isolates of *N. gonorrhoeae*

Patient	Isolate no.	MIC (mg/liter) of penicillin/β-lactamase	MIC (mg/liter) of tetracycline/*tetM*	MIC (mg/liter) of:			
				Spectinomycin	Ceftriaxone	Ciprofloxacin	Ofloxacin
A	A1	0.125/negative	64/positive	16	≤0.007	≤0.007	0.03
	A2	0.25/negative	4/negative	8	≤0.007	≤0.007	0.015
B	B1	1/negative	128/positive	16	≤0.007	≤0.007	0.03
	B2	1/negative	128/positive	16	≤0.007	0.125	2
C	C1	0.5/negative	32/positive	16	≤0.007	≤0.007	≤0.007
	C2	0.5/negative	16/positive	16	≤0.007	≤0.007	0.015
D	D1	8/positive	32/positive	16	≤0.007	≤0.007	≤0.007
	D2	4/positive	32/positive	16	≤0.007	≤0.007	0.015
E	E1	0.5/negative	2/negative	16	≤0.007	≤0.007	0.015
	E2	1/negative	1/negative	16	≤0.007	≤0.007	0.03
F	F1	0.25/negative	32/positive	32	≤0.007	≤0.007	0.015
	F2	0.25/negative	32/positive	16	≤0.007	≤0.007	0.015
G	G1	0.25/negative	16/positive	16	≤0.007	≤0.007	≤0.007
	G2	0.25/negative	32/positive	16	≤0.007	≤0.007	0.015

TABLE 3. Comparison of pre- and post-treatment *N. gonorrhoeae* isolates from seven women by four typing methods

Patient	Isolate no.	Result of typing system:				Identical isolates
		Antibiogram	Auxotype[a]	Serotype	opa type	
A	A1	Different	NR	IA-6	Different	No
	A2		PRO	IB-2		
B	B1	Different	ARG	IA-6	Different	No
	B2		NR	IB-7		
C	C1	Same	NR	IB-8	Different	No
	C2		NR	IB-3		
D	D1	Same	NR	A-NT	Same	Yes
	D2		NR	A-NT		
E	E1	Same	PRO	IB-3	Different	No
	E2		PRO	IB-3		
F	F1	Same	NR	IB-3	Different	No
	F2		NR	IB-6		
G	G1	Same	NR	IB-3	Different	No
	G2		NR	IA-6		

[a] NR, not requiring; PRO, proline requiring; ARG, arginine requiring.

types. Isolate B2 was associated with an increase in MIC compared to B1, exhibiting reduced susceptibility to ciprofloxacin and resistance to ofloxacin. Isolates B1 and B2 also belonged to different A/S classes and opa types. The paired isolates from patient D were indistinguishable by antibiogram, A/S typing, and opa typing. Isolates E1 and E2 were indistinguishable by antibiogram and A/S typing but differed by genotyping. The individual isolates of pairs C, F, and G differed by phenotype and genotype.

DISCUSSION

In this study, six of seven patients, who presented 8 to 10 days following treatment, were shown to be infected with gonococcal isolates that differed significantly from their primary isolate. This was despite the susceptibility of the initial isolates to ciprofloxacin that was administered as a directly observed single dose. Hence, the most likely explanation is reinfection.

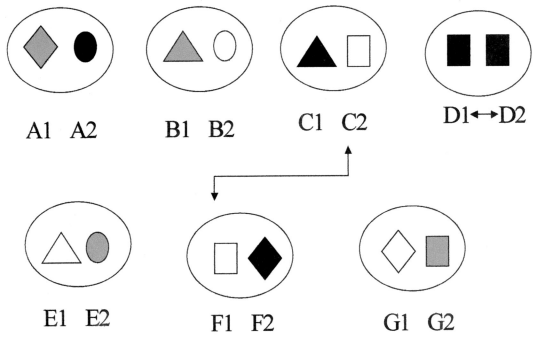

FIG. 1. Opa types of seven pairs (pre- and post-treatment isolates) of *N. gonorrhoeae* isolates. Double-headed arrows indicate identical opa types.

This illustrates the failure of efforts to control the spread of STIs.

The phenotypes of the paired isolates from patients A and B were different (Table 3). The isolates in these pairs also differed in their opa type. Theoretically, the subsequent isolate from patient B could be the result of failure of treatment of a mixed population of gonococci. B1 was susceptible to ciprofloxacin and would therefore have been eradicated by ciprofloxacin, while B2, with its intermediate susceptibility to ciprofloxacin, may have persisted. Isolate A1 differed from A2 both phenotypically and genotypically in its susceptibility to tetracycline. Both these isolates were susceptible to ciprofloxacin. Selection of A2 from a mixed infection is highly unlikely because A2 was susceptible to tetracyclines while A1 was resistant. A2 therefore most probably represents reinfection.

Apart from patient D, the opa types of pairs of isolates from patients with comparable antibiograms (C, D, E, F, and G) were different. This indicates that antibiograms have little value in establishing relatedness between isolates in areas where there is no resistance or, as in our area, widespread acquired resistance to some antibiotics. Both situations lead to similar antibiograms of all isolates.

Like the antibiograms, auxotyping also had little discriminative value within these 14 isolates. This is the result of the large number of nonrequiring isolates. Serotyping, on the other hand, discriminated very well within this group, except for one pair (from patient E), which was identical by serotyping but differed in genotype (Table 3).

The opa types of the initial isolates differed from those of the subsequent isolates in all but pair D. The high degree of unrelatedness of isolates from one individual within a time span of 8 to 10 days suggests rapid reinfection or infection with multiple isolates at baseline. Reports on the prevalence of coinfection with a mixed population of gonococci have varied from not being present to being common (11, 12, 14). However, since all but one isolate (B2) were highly susceptible to ciprofloxacin, coinfection is unlikely, since the drug would have eradicated the susceptible mixed population of *N. gonorrhoeae.* Another possible explanation for the discrepancy is short-term genetic instability (14). This is an unlikely explanation for our observations since the interval between sampling was short and opa typing revealed multiple different bands between the paired isolates. Opa typing is highly discriminatory yet is able to identify linked isolates in a transmission chain (15). Therefore, these isolates most probably represent reinfection with a different strain. This strain could originate from the same untreated partner if this partner was coinfected with more than one strain. We have no information about the frequency of infection with multiple strains in our population. This needs further investigation. The alternative is that this reinfection came from a different partner. This seems to be a likely explanation because the only patient who was reinfected with the same strain (patient D) had, with RF = 0.4, the lowest risk in this small group of women. This was despite the fact that patient D was the only one with multiple current partners. This can be explained by one partner being a migrant laborer and one being a resident in the area. The role of the RF (i.e., the number of lifetime partners divided by the number of years of sexual activity) in defining the possibility of acquiring an STI needs further validation in experiments with a larger group.

Our conclusion that these women were probably reinfected by different partners is supported by the observation that in our area, as many women as men are HIV-1 positive in discordant couples (M. Lurie, S. S. Abdool Karim, and A. W. Sturm, Abstr. Guide Thirteenth Meet. Int. Soc. Sex. Transm. Dis. Res., p. 52, 1999). This implies that these women have acquired their HIV infection from a sexual partner other than the stable partner.

Isolates C2 and F1 were indistinguishable by opa typing with both restriction enzymes and by antibiogram and A/S class. The presence of gonococci with indistinguishable opa types is a good indicator that the individuals from whom they were recovered were sexual partners or part of a short chain of disease transmission (10, 15). Therefore, this opa type result for isolates from patients C and F may indicate an undisclosed or unknown sexual linkage between the individuals. The frequency of women having sex with women is unknown in our setting but is believed to be low. Therefore, our data suggest that these women may have a common partner or are part of a common network.

Interestingly, all women, with the exception of patient D, admitted to only one current sexual contact. The typing patterns of their isolates, however, suggest otherwise. The results of this study, in conjunction with our previous report (8), imply that about 20% of female STI clinic attendees become rapidly reinfected following successful drug treatment for *N. gonorrhoeae* infection. Even more alarming is the diversity of the isolates within the pairs, suggesting rapid reinfection within the partnerships.

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