ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1985, p. 393–396
0066-4804/85/090393-04$02.00/0

Vol. 28, No. 3

Antimicrobial Susceptibility Testing and Phenotyping of *Neisseria gonorrhoeae* Isolated from Patients with Ophthalmia Neonatorum in Nairobi, Kenya

ROBERT C. BRUNHAM,[1,2]* LIEVE FRANSEN,[3] FRANCIS PLUMMER,[1,2] PETER PIOT,[3] LESLIE SLANEY,[2] SOLGUN BYGDEMAN,[4] AND HERBERT NSANZE[5]

Departments of Medicine[1] and Medical Microbiology,[2] University of Manitoba, Winnipeg, Manitoba, Canada; Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium[3]; Department of Clinical Bacteriology, Karolinska Institute at Sodersjukhuset, Stockholm, Sweden[4]; Department of Medical Microbiology, University of Nairobi, Nairobi, Kenya, East Africa[5]

Received 2 May 1985/Accepted 6 June 1985

Antimicrobial susceptibility testing, auxotyping-serotyping, and plasmid analysis were performed on 41 ocular isolates, 7 nasopharyngeal isolates, and 18 cervical isolates of *Neisseria gonorrhoeae* obtained during a recent treatment trial of gonococcal opthalmia neonatorum in Nairobi, Kenya. Fourteen distinct serovar-auxotype patterns were observed with IB-1/Pro$^-$ strains which accounted for 59% of the isolates. Infection with multiple types of gonococci appeared to occur in 22% of the mothers since 4 of 18 paired maternal cervical and neonatal ocular isolates had mismatched serovar-auxotype patterns. Among 10 treatment failure isolates only 1 had a mismatched serovar-auxotype pattern. Six (15%) of the ocular isolates were penicillinase-producing *N. gonorrhoeae* (PPNG). Five had the 4.4-megadalton (Md) β-lactamase plasmid and one had the 3.2-Md β-lactamase plasmid. The 24.5-Md plasmid was found in 5 of 6 PPNG strains and in 8 of 35 non-PPNG strains ($P < 0.02$). For most antimicrobial agents, PPNG and non-PPNG strains showed similar patterns of susceptibility. Ceftriaxone was the most active of the antibiotics tested, with all strains having an MIC ≤0.06 mg/liter. Among non-PPNG strains, 15 (43%) had a penicillin MIC ≥ 2 mg/liter and were considered intrinsically resistant to penicillin. Overall, non-PPNG intrinsically resistant strains had greater resistance to other antibiotics than did non-intrinsically resistant strains ($P \leq 0.006$). The Mtr phenotype was found in 53% of these strains.

Ophthalmia neonatorum due to *Neisseria gonorrhoeae* is a major neonatal infection in Nairobi, Kenya, where eye prophylaxis at birth is rarely used. Approximately 5% of newborns at one Nairobi hospital develop gonococcal ophthalmia, and 12% of mothers have cervical gonococcal infection (H. Nzanse, unpublished data). We have recently reported the results of treatment of 117 infants with gonococcal ophthalmia neonatorum with single-dose kanamycin therapy (2). Since no study has yet reported on the phenotypic characteristics of gonococcal strains isolated from eyes, we evaluated these strains for auxotype, protein I serovar, and cell envelope phenotype. Additionally, since penicillinase-producing *N. gonorrhoeae* (PPNG) have been recently introduced and spread throughout Kenya (F. A. Plummer, L. D'Costa, H. Nsanze, L. Slaney, W. DeWitt, J. Knapp, J. Dillon, W. Albritton, and A. R. Ronald, *in Immunobiology of Neisseria gonorrhoeae*, in press), we studied these strains for plasmid content and for the antimicrobial susceptibility to penicillin and several other antibiotics.

MATERIALS AND METHODS

Strains of *N. gonorrhoeae*. All strains were received from Antwerp, Belgium, in a lyophilized state. Ocular isolates were available from 41 neonates. Nasopharyngeal isolates were also available from seven of these neonates. Six isolates which represented neonatal treatment failures were also available (4 were isolated from the eyes and 2 were from the nasopharynx). Cervical isolates were available from 18 mothers. Subsequent isolates from four of these mothers who failed therapy were also available.

Microbiologic studies. Upon receipt, all strains were confirmed to be *N. gonorrhoeae* by typical colony morphology; typical gram-stain morphology; oxidase positivity; production of acid from glucose; and negative reactions with maltose, sucrose, and *o*-nitrophenyl-β-D-galactopyranoside by the Minitek method (9). All strains were tested for β-lactamase production by nitrocefin hydrolysis Becton Dickinson and Co., Mississauga, Ontario, Canada).

Auxotyping. Auxotyping was performed by the method of Hendry and Stewart (4). Nutritional requirements for proline (Pro$^-$), ornithine (Orn$^-$), and arginine (Arg$^-$) were determined. Strains requiring none of these amino acids were identified as nonrequiring (NR).

Protein I typing. Strains were categorized as protein IA or protein IB in a coagglutination assay with a panel of monoclonal antibodies to protein I by the classification system of Knapp et al. (6). Within each protein I group, strains were subclassified as to serovar and, available when designations were assigned, they were by the monoclonal reaction pattern specified by Knapp et al. (6).

Determination of cell envelope phenotype. Susceptibility to erythromycin and Triton X-100 were used to identify provisionally the cell envelope phenotype of strains (10). Strains were defined as wild type if they had erythromycin MICs of 0.12 to 1 mg/liter, Mtr phenotypes if they had erythromycin MICs ≥ 2 mg/liter and Triton X-100 MICs ≥ 2 g/liter, Env phenotypes if they had erythromycin MICs < 0.06 mg/liter and Triton X-100 MICs < 0.5 g/liter.

Antimicrobial susceptibility testing. Agar dilution suscepti-

* Corresponding author.

TABLE 1. Serovar and auxotype of the four mismatched neonatal ocular and maternal cervical paired isolates of *N. gonorrhoeae*

Case no.	Serovar-auxotype of isolates from:	
	Maternal cervix	Neonatal eye
1	IB-1[a]/Pro$^-$	IA-3/NR
2	IB-1/Pro$^-$	IA-6/Pro$^-$ Orn$^-$
3	IB-4/NR	IB-1/Pro$^-$
4	IB-1/NR	IA-X[b]/Pro$^-$

[a] The serovar designation according to Knapp et al. (6).
[b] IA-X, protein IA-bearing strain with a monoclonal reaction pattern not currently specified by the system of Knapp et al. (6).

bility tests were performed on chocolate agar (5). Five antibiotics were tested using doubling dilutions of the drugs in the ranges shown (in milligrams per liter): penicillin (128 to 0.008), ceftriaxone (0.06 to 0.0005), erythromycin (4 to 0.15), tetracycline (4 to 0.03), and thiamphenicol (32 to 0.12). The aminocyclitol antibiotics, kanamycin, gentamicin, and spectinomycin, were tested at intervals of 2 mg between 2.0 and 32 mg/liter. Susceptibility to Triton X-100 was determined for concentrations between 4 and 0.5 g/liter. Overnight chocolate agar cultures of the gonococci were suspended in broth and diluted so that Steer's replicator inoculation of the plates delivered ca. 5×10^3 CFU at the point of contact. The dried plates were incubated at 35°C in a humid 5% CO_2 atmosphere.

Plasmid analysis. Plasmids were collected from gonococcal strains which were grown overnight on chocolate agar and lysed with sodium dodecyl sulfate and EDTA (8). The proteins were removed by phenol extraction of the salt-cleared lysates. The DNA, precipitated with cold ethanol, was redissolved and visualized under long wave UV after electrophoresis in 0.7% agarose and ethidium bromide staining.

Statistical analysis. Chi-square and Fisher's exact tests were used for analysis of sample proportions. Differences in antimicrobial susceptibilities were compared with a two-tailed Student's *t* test of geometric mean MICs.

RESULTS

Serotyping-auxotyping of isolates. Among the 41 pretreatment ocular isolates of *N. gonorrhoeae,* the following serovar-auxotype patterns were observed: 24 IB-1/Pro$^-$, 2 IB-1/NR, 2 IB-3/Pro$^-$, 1 IB-3/NR, 1 IB-4/Pro$^-$, 2 IB-4/NR, 1 IA-3/NR, 1 IA-4/NR, 1 IA-5/NR, 2 IA-6/Pro$^-$, and 1 IA-10/Pro$^-$ Orn$^-$. Among the six β-lactamase-positive *N. gonorrhoeae* strains, three were IB-1/Pro$^-$, two were IB-1/NR, and one was IA-4/NR. Among 18 matched pairs of maternal cervical and neonatal ocular isolates, the serovar-auxotype pattern was identical in 14. The nonmatched paired ocular-cervical isolates are shown in Table 1.

Among eight paired neonatal ocular-nasopharyngeal isolates, five were concordant for the serovar-auxotype pattern. Among 10 neonatal or maternal treatment failure isolates, 9 had concordant serovar-auxotype reactions between pre- and posttreatment isolates. The single exception differed in only one monoclonal antibody reaction.

Phenotypic characteristics and plasmid profiles of β-lactamase-positive and -negative strains of *N. gonorrhoeae*. The 6 β-lactamase-producing strains were compared with 35β-lactamase-negative strains (Table 2).Wild-type cell envelope was observed in 1 of 6 β-lactamase-positive strains versus 17 of 35 β-lactamase-negative strains ($P > 0.05$). There were no significant differences in the proportion of strains with protein IA or IB serotypes, in the Mtr or Env cell envelope phenotypes, or in the proportion of the 2.6-megadalton (Md) plasmid between the two groups. Among the β-lactamase-producing strains, five had the 4.4-Md β-lactamase plasmid and one had the 3.2-Md plasmid. A total of five strains had the 24.5-Md mobilizing plasmid, whereas 8 of the 35 β-lactamase-negative strains had the plasmid ($P < 0.02$).

Antimicrobial susceptibility testing. For antibiotics other than penicillin, PPNG strains had comparable susceptibility results to non-PPNG strains (Table 3). Non-PPNG strains were more susceptible to spectinomycin ($P = 0.04$) and gentamicin ($P = 0.04$) than were PPNG strains, but these differences were small.

Among the 35 non-PPNG strains, 15 had penicillin MICs ≥ 2 mg/liter. These strains were thus intrinsically resistant (IR) to penicillin. For antibiotics other than spectinomycin, IR strains were more resistant to all antibiotics tested than were non-IR strains ($P \leq 0.006$ for all comparisons).

Characteristics of IR strains of *N. gonorrhoeae*. Fifteen IR strains were compared with the 20 strains which had a penicillin MIC ≤ 1 mg/liter (Table 4). The cell envelope of the IR strains differed in three significant ways from penicillin-susceptible strains. Protein IB serovars were found in all 15 IR strains compared with 15 of 20 in susceptible strains ($P = 0.048$), the Mtr phenotype was found in 8 IR strains versus 3 in susceptible strains ($P = 0.02$), and the Env phenotype was found in no IR strain versus 7 in susceptible strains ($P = 0.012$). There was no difference in the proportion of resistant and susceptible strains harboring the 2.6-Md plasmid (15 of 15 IR strains versus 19 of 20 susceptible strains) or the 24.5-Md plasmid (4 of 15 IR strains versus 4 of 20 susceptible strains).

DISCUSSION

Serovar-auxotyping proved useful in characterizing gonococcal strains in this study. The major serovar-auxotype IB-1/Pro$^-$ strains accounted for 59% of the ocular isolates, although 14 distinct serovar-auxotype patterns were discerned. These typing features were relatively stable in

TABLE 2. Phenotypic characteristics and plasmid profiles of β-lactamase-positive and -negative ocular isolates of *N. gonorrhoeae*

Phenotypes and plasmids	No. of isolates with the indicated β-lactamase-producing ability:	
	Positive ($n = 6$)	Negative ($n = 35$)
Phenotype		
Protein IA	1	5
Protein IB	5	30
Mtr	2	11
Env	3	7
Plasmid		
2.6 Md		27
2.6 Md + 24.5 Md		7
24.5 Md		1
2.6 Md + 3.2 Md	1	
2.6 Md + 4.4 Md + 24.5 Md	5	

TABLE 3. Antimicrobial susceptibility testing

Antibiotic	MIC (mg/liter) for[a]:											
	PPNG (n = 6)			Penicillin susceptible (n = 20)			Penicillin resistant (n = 15)			Penicillin susceptible and resistant (n = 35)		
	50%	90%	Range	50%	90%	Range	50%	90%	Range	50%	90%	Range
Penicillin	22.6	128.00	8–28	0.25	0.93	0.008–1.0	4.0	4.0	2–4	0.5	4	0.008–4.0
Ceftriaxone	0.008	0.015	0.004–0.015	0.006	0.015	0.0005–0.15	0.03	0.06	0.008–0.06	0.015	0.06	0.0005–0.06
Erythromycin	0.042	2.00	0.03–2.0	0.173	2.00	0.015–2.0	2.0	2.64	1.0–4.0	1.00	2.00	0.015–4.0
Tetracycline	2.8	4.5	2–4.5	2.0	3.73	1.0–4.5	4.5	4.5	4.5–4.5	2.0	4.5	1–4.5
Spectinomycin	14.97	16.00	14–16	14.00	15.79	10–18	14.0	16.0	10–16	14.0	16.0	10–18
Kanamycin	14.0	32.00	14–32	14.0	18.0	10–20	18.0	20.0	14–20	16.0	20.0	10–20
Gentamicin	8.0	10.00	6–10	6.0	7.77	4–8	8.0	8.0	6–8	6.0	8.0	4–8
Thiamphenicol	1.4	2.0	0.12–2.0	1.0	2.0	0.5–4.0	2.0	4.0	2.0–4.0	2.0	4.0	0.5–4.0

[a] 50% and 90%, MIC for 50 and 90% of the strains, respectively.

infection chains and in the same host with treatment failure. All treatment failure isolates were of the same or nearly identical auxotype-serovar pattern as the original pretreatment isolate. However, 4 of 18 maternal-neonatal strain pairs were discordant in serovar-auxotype reactions. The discrepancies reflected major antigenic or nutritional differences or both between maternal (Table 1) and neonatal isolates and are unlikely to be accounted for by minor variations in test performance. Rather, these results suggest that maternal infection with multiple types of gonococci has occurred. This hypothesis is consistent with the observation that two of seven strains isolated from neonates with both ocular and nasopharyngeal infections were also discordant for serovar-auxotype reactions. Prospective evaluation of infected mothers for multiple types of gonococcal strains and subsequent transmission of one of these types to the newborn infant is required to validate this hypothesis.

Infection with multiple strains of gonococci is more likely to occur in individuals who are members of a core group of high-frequency transmitters of gonococcal infection (11). In our study, most mothers resided in an area of Nairobi where a high prevalence of gonorrhea is known to occur (Plummer et al., in press). Thus, we suspect that many of the mothers in our study were members of a high-frequency transmitter core group. Infection with multiple strains of gonococci may be particularly important in the overall ecology of gonococci since exchange of plasmid or chromosomal DNA may occur under these in vivo circumstances and allow for the development of phenotypic change or in the acquisition of antimicrobial resistance genes.

An interesting finding in our study was the multiple mechanisms for penicillin resistance observed among these strains. These mechanisms included two plasmid- and at least two chromosome-mediated mechanisms. Of the 41 strains 6 (14%) produced β-lactamase. Five were specified by a 4.4-Md plasmid, and 1 was specified by a 3.2-Md plasmid. This relatively low prevalence of PPNG is no longer the case since in more recent studies 60% of strains isolated from neonates with gonococcal ophthalmia in Nairobi produce β-lactamase (H. Nsanze, unpublished data).

TABLE 4. Phenotypic characteristics and plasmid profiles of IR *N. gonorrhoeae*

Phenotype or plasmid	No. of IR isolates with the following penicillin MICs (mg/liter):		P
	<2 (n = 20)	≥2 (n = 15)	
Protein IB serogroup	15	15	0.048
Mtr phenotype	3	8	0.02
Env phenotype	7	0	0.012
2.6-Md plasmid	19	15	NS[a]
24.5-Md plasmid	4	4	NS

[a] NS, Not significant.

The six PPNG strains in this study did not differ significantly in terms of protein I serovar or auxotype from nonproducing strains. The 24.5-Md mobilizing plasmid was found significantly more often in PPNG strains than in non-PPNG strains (5 of 6 versus 8 of 35; $P < 0.02$). The 90% MICs for most antibiotics were similar for PPNG and non-PPNG strains. Overall, ceftriaxone was the most active of the antibiotics tested.

Most PPNG strains in this study had a non-wild-type cell envelope phenotype. In particular, the Env phenotype was detected in 50% of PPNG strains compared with 20% of non-PPNG strains, although this did not reach statistical significance in this small sample of strains. Eisenstein et al. (1) have also observed that the Env phenotype is frequently found in Far Eastern PPNG strains. We consider it possible that Env mutants may be more competent for plasmid or chromosomal transformation, a finding which if true could give selective advantage to these hypersensitive strains in nature and may facilitate the dissemination of the β-lactamase-specifying plasmid in populations of gonococci. Transformations or conjugation frequences for the Env, Mtr, and wild-type phenotypes have yet to be reported.

Among the 35 non-PPNG strains, 15 strains were intrinsically resistant to penicillin with MICs ≥ 2 mg/liter. The Mtr phenotype was found in 8 of the 15 IR strains. Mtr strains have been thought to be permeability mutants and have been characterized by nonspecific resistance to multiple antibiotics, hydrophobic dyes, and detergents (7). Mtr strains possess many differences when compared with other strains of gonococci, including differences in the phospholipid heads of outer leaflet lipids (7), a sevenfold increase in the quantity of a 52,000-molecular-weight outer membrane protein, and an increase in the extent of peptidoglycan cross-linking (3). In North America, such strains have been suggested to have selective advantage in environments such as the rectum in which inhibitory hydrophobic molecules are found in abundance (10). Overall in our study, 13 (32%) of 41 ocular isolates and 6 (33%) of 18 maternal cervical isolates were found to have the Mtr phenotype. This high prevalence of the Mtr phenotype among neonatal ocular and maternal

cervical isolates is similar to that noted for rectal isolates from homosexual men in Seattle, Washington (14 of 58 isolates), but is much higher than that noted in cervical isolates (2 of 39) from women in the same study (10). Ecological factors within the maternal cervix which serve to maintain this high prevalence of the Mtr phenotype in Nairobi require further study.

LITERATURE CITED

1. **Eisenstien, B., and F. Sparling.** 1978. Mutations to increased antibiotic sensitivity in naturally occurring gonococci. Nature (London) **271**:242–244.
2. **Fransen, L., L. D'Costa, A. Ronald, H. Nsanze, R. Brunham, and P. Piot.** 1984. Single-dose kanamycin therapy of gonococcal ophthalmia neonatorum. Lancet **ii**:1234–1237.
3. **Guymon, L., D. Walstad, and F. Sparling.** 1978. Cell envelope alterations in antibiotic-sensitive and -resistant strains of *Neisseria gonorrhoeae*. J. Bacteriol. **136**:391–401.
4. **Hendry, A., and I. Stewart.** 1979. Auxanographic grouping and typing of *Neisseria gonorrhoeae*. Can. J. Microbiol. **25**:512–521.
5. **Jaffe, H., J. Biddle, C. Thornsberry, R. Johnson, R. Kaufman, G. Reynolds, P. Wiesner, and the Cooperative Study Group.** 1976. National gonorrhea therapy monitoring study. In vitro antibiotic susceptibility and its correlation with treatment results. N. Engl. J. Med. **294**:5–9.
6. **Knapp, J., M. Tam, R. Nowinski, K. Holmes, E. Sandstrom.** 1984. Serological classification of *Neisseria gonorrhoeae* with use of monoclonal antibodies to gonococcal outer membrane protein. J. Infect. Dis. **150**:44–48.
7. **Lysko, P., and S. Morse.** 1981. *Neisseria gonorrhoeae* cell envelope: permeability to hydrophobic molecules. J. Bacteriol. **145**:946–952.
8. **Meyers, J., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. A simple agarose gel electrophoresis method for identification and characterization of plasmid DNA. J. Bacteriol. **124**:1529–1537.
9. **Morse, S., and L. Barenstein.** 1976. Adaptation of the Minitek system for the rapid identification of *Neisseria gonorrhoeae*. J. Clin. Microbiol. **3**:8–13.
10. **Morse, S., P. Lysko, L. McFarland, J. Knapp, E. Sandstrom, C. Critchlow, and K. Holmes.** 1982. Gonococcal strains from homosexual men have outer membranes with reduced permeability to hydrophobic molecules. Infect. Immun. **37**:432–438.
11. **Yorke, J., H. Hethcote, and A. Nold.** 1978. Dynamics and control of the transmission of gonorrhea. Sex. Trans. Dis. **5**:51–56.