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Molecular and Phenotypic Characterization of Penicillinase-Producing *Neisseria gonorrhoeae* from Canadian Sources

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The incidence of penicillinase-producing *Neisseria gonorrhoeae* (PPNG) infections has increased in Canada during the past 2 years. Most of these cases were imported from abroad. The PPNG strains from these cases were characterized with respect to susceptibility to 11 antibiotics, auxotype, and plasmid content. Rosaramicin and cefuroxime proved to be the most potent of the antibiotics tested. The molecular characterization of the isolates indicated that all carried a 2.6-megadalton cryptic plasmid. Most of the PPNG isolates (87%) harbored a 4.5-megadalton penicillinase-producing plasmid, whereas only 13% harbored the 3.2-megadalton penicillinase-producing plasmid. In those cases where contact tracing was possible, the correlation linking strains of Far Eastern etiology with carriage of the 4.5-megadalton plasmid was upheld. The penicillinase-producing strains were typed auxanographically in either the proline-requiring (57%) or prototrophic groups (42%). Substrate hydrolysis profiles and analytical isoelectric focusing of crude β-lactamase extracts of several isolates has reconfirmed that these strains elaborate a type TEM-1 enzyme. Several of the penicillinase-producing plasmids were also examined for plasmid stability.

The development of plasmid-mediated penicillin resistance in isolates of *Neisseria gonorrhoeae* (1, 27, 30), with its subsequent worldwide prevalence (35, 36), has altered our perspectives on the acquisition of other plasmid-determined antibiotic resistances in gonococci and on the treatment of gonococcal infections. The monitoring and characterization of penicillinase production in *N. gonorrhoeae* has shown that the penicillinase plasmids are of two distinct sizes (28, 31) and that they are closely related to plasmids from the genus *Haemophilus* (10, 38). The β-lactamase gene on these plasmids comprises about 40% of the ampicillin transposon Tn*2* (31). Tn*2*, which encodes for the production of a type TEM β-lactamase is present on a large number of plasmids of differing incompatibility groups (16). Therefore, the acquisition of a type TEM β-lactamase by gonococci represents an extension of the intergeneric range of this penicillin transposon.

Surveillance of plasmid-mediated penicillin resistance in *N. gonorrhoeae* strains isolated in Canada began in 1976. Most penicillinase-producing *N. gonorrhoeae* (PPNG) strains isolated in Canada are sent to our laboratory at the Laboratory Center for Disease Control for phenotypic confirmation and plasmid characterization. In the present study, we report on the 69 isolates received, to date, by our laboratory. We have tested their susceptibilities to 11 antimicrobial agents, examined their β-lactamase hydrolysis profiles, and characterized the plasmids they contain. The stability of the penicillinase-producing plasmids in *N. gonorrhoeae* host strains and in *Escherichia coli* transformants was also examined.

MATERIALS AND METHODS

Strains. A total of 69 individual PPNG isolates from Canadian sources, submitted to our laboratory either by the provincial Ministries of Health or by private laboratories for confirmation as PPNG and for plasmid analysis, were used in this study. Five additional strains (GC1-15 to GC1-19) were obtained from E. H. Sng, Ministry of Health, Singapore. The reference β-lactamase-positive isolate GC1-5, carrying a prototype 4.5-megadalton penicillinase plasmid, was kindly supplied by C. Thornsberry, Centers for Disease Control, Atlanta, Ga. (CDC 76-061782). Reference PPNG strain GC1-31, harboring a 3.2-megadalton penicillinase plasmid, and World Health Organization reference strains III, V, and VII, which were used in antimicrobial susceptibility tests, were supplied by A. Reyn, Copenhagen, Denmark. *E. coli* 490 *recA hsdS* was used in all transformation experiments. Reference plasmid-containing strains which produced type TEM β-lactamases (pRP4 and pR6K) were provided by V.

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Minimum inhibitory concentration and auxotype determinations. All isolates were subcultured on GC medium base (Difco Laboratories) supplemented with 1% Kellogg defined supplement (19) and 5 μg of ampicillin (for β-lactamase-positive isolates) per ml. Antibiotic-containing medium for minimum inhibitory concentration determinations consisted of GC medium base containing 1% defined supplement and doubling dilutions of antibiotic to give the following final concentrations: penicillin G and ampicillin (Ayerst Laboratories), 0.008 to 128.0 μg/ml; erythromycin (Sigma Chemical Co.), 0.032 to 2.0 μg/ml; rosaramicin (Schering Corp.) 0.004 to 2.0 μg/ml; spectinomycin (The Upjohn Co.), 2.0 to 32.0 μg/ml; tetracycline hydrochloride (Bristol Laboratories), 0.032 to 4.0 μg/ml; thiamphenicol (Zambon) and chloramphenicol (Sigma), 0.016 to 8.0 μg/ml; spiramycin (Poulenc), 0.063 to 16.0 μg/ml; cefuroxime (Glaxo Laboratories, Ltd.), 0.002 to 0.5 μg/ml; and cefoxitin (Merck & Co.), 0.002 to 1.0 μg/ml.

Auxanographic typing of isolates was determined by using the method of Hendry and Stewart (17). Media for antibiotic susceptibility and auxotype testing were inoculated as described previously (8). Inoculated plates were incubated for 24 to 36 h in a humid environment, with 5% CO_2 at 35°C. The minimum inhibitory concentration was taken as that concentration of antibiotic which completely inhibited growth; with equivocal readings, the presence of one to nine colonies was accepted as an endpoint.

β-Lactamase assays and analytical isoelectric focusing. Isolates were initially screened for β-lactamase activity with the chromogenic cephalosporin substrate (25) nitrocefin (Glaxo Laboratories, Ltd.). Substrate hydrolysis profiles were determined by the macroiodometric method as described by Ross and O'Callaghan (34). Isoelectric points were determined as described by Matthew et al. (22).

Characterization of plasmid DNA and transformation procedures. Plasmid deoxyribonucleic acid (DNA) from *N. gonorrhoeae* isolates was prepared for agarose gel electrophoresis either by isolating the DNA by cesium chloride-ethidium bromide ultracentrifugation, using a modification of the technique employed by Engelkirk and Schoenhard (11), or by using the cell lysis screening technique of Sox et al. (37). Agarose gel electrophoresis of plasmid DNA was carried out as described by Meyers et al. (24). Reference plasmid DNA (pBR 322, ColE1, RSC11, and pKN102) was kindly supplied by G. Bezanson (Laboratory Center for Disease Control, Ottawa, Ontario, Canada).

Transformation of *E. coli* 490 with plasmid DNA from *N. gonorrhoeae* isolates was carried out as described by Lederberg and Cohen (21). Transformed cells expressing resistance to ampicillin were selected on GC medium base supplemented with 10.0 μg of ampicillin per ml. Cleared lysates of transformed *E. coli* 490 were prepared by using the method of Clewell and Helinski (7), except that Triton X-100 was used to achieve lysis.

Stability studies. PPNG isolates GC1-5, GC1-14, and GC1-31, as well as their respective β-lactamase-positive *E. coli* 490 transformants (designated EC1-5, EC1-14, and EC1-31), were examined for their ability to maintain ampicillin resistance under nonselective conditions. A single colony from each strain, which was β-lactamase positive, was subcultured onto GC medium base agar plates without antibiotic. The strains were then nonselectively subcultured onto fresh agar plates for 10 consecutive days. Each day, a cell suspension was prepared in 0.7% Casamino Acids (Difco Laboratories) such that the optical density (wavelength, 590 nm; Bausch and Lomb Spectronic 20) was adjusted to 0.75. This corresponded to a concentration of approximately 5×10^8 bacteria per ml. Appropriate dilutions of the suspension were plated, in duplicate, on GC medium agar base both with and without ampicillin (5 and 10 μg/ml). The stability of ampicillin resistance was expressed as the percent of the ratio of the viable count on antibiotic-containing medium to the viable count on antibiotic-free medium.

RESULTS

Epidemiology of PPNG in Canada. Over the past 2 years, the incidence of PPNG infections in Canada has increased appreciably. Eighty-three percent (57/69) of all PPNG isolates submitted to the Laboratory Center for Disease Control for confirmation were received during 1979 to 1980. By way of comparison, only one isolate was received during 1976 (the year Canadian surveillance of PPNG infections was first initiated), five were isolated in 1977, and six were isolated in 1978.

The geographic distribution of the PPNG isolates coupled with data pertaining to the origin of infection (Table 1) indicate that most PPNG infections were imported from other countries; the majority were imported from Asia. Twelve of the sixteen cases originating in Canada were contacts of persons infected abroad, indicating that a pool of PPNG has not, as yet, been established in this country.

Antibiotic susceptibility and β-lactamase enzymes of PPNG strains. The susceptibilities of 55 PPNG strains to 11 antibiotics was examined (Table 2). As might be expected, the strains were resistant to high concentrations of penicillin and ampicillin and were susceptible to clinically applicable concentrations of spectinomycin. Among the other antibiotics tested, the most potent was rosaramicin, followed by cefuroxime, erythromycin, cefoxitin, tetracycline, thiamphenicol, spiramycin, and chloramphenicol. Rosaramicin, a macrolide antibiotic, proved to be the most potent, on a weight basis, and was slightly more effective than cefuroxime.

Substrate hydrolysis profiles (eight isolates tested) coupled with isoelectric focusing of crude lysates (four isolates) confirmed previous reports (12, 27, 39, 41) that PPNG produced type TEM-1 β-lactamase enzymes. The eight gonococcal isolates tested showed high hydrolysis activity

TABLE 1. *Geographic distribution and origin of infections of PPNG strains isolated in Canada*[a]

Province of isolation	Origin of infection (no. of isolates)							
	Canada	Asia	Africa	Caribbean and Mexico	United States	No information	Total	%
Nova Scotia	—[b]	1	—	—	1	—	2	2.9
Quebec	2	—	—	3	—	2	7	10.2
Ontario	10	11	1	2	1	4	29	42.0
Manitoba	—	1	—	—	—	—	1	1.5
Alberta	4	9	—	—	2	—	15	21.7
British Columbia	—	6	1	—	2	6	15	21.7
Total	16	28	2	5	6	12	69	
%	23.2	40.6	2.9	7.2	8.7	17.4		100

[a] Represents strains submitted to the Laboratory Center for Disease Control for confirmation as PPNG. Period includes 1976 to December 1980.
[b] —, Indicates zero incidence.

TABLE 2. *Susceptibilities of 55 PPNG isolates to 11 antibiotics*

Antibiotic	% Inhibition at concn (μg/ml)[a]										
	≤0.16	0.063	0.25	0.5	1.0	2.0	4.0	8.0	16.0	128.0	>128.0
Penicillin										25.5	100
Ampicillin										18.2	100
Cefuroxime	14.6	56.4	85.5	96.6[b]							
Cefoxitin				29.1	72.7[b]						
Erythromycin		18.2	38.2	58.2	76.4	90.9	100				
Rosaramicin	23.6	58.2	89.1	98.2	100.0						
Spiramycin			7.3	27.3	38.2	54.6	72.7	96.6	98.2[b]		
Chloramphenicol				12.7	18.2	32.7	74.6	98.2[b]			
Thiamphenicol			12.7	14.6	52.7	78.2	96.6	100			
Tetracycline			5.5	38.2	69.1	81.3	100				
Spectinomycin								27.3	98.2		

[a] Doubling dilutions of antibiotic were prepared from 0.004 to 128 μg/ml. Not all results are indicated.
[b] Final dilution tested.

against penicillin, ampicillin, hetacillin, and cephaloridine. Carbenicillin and oxacillin were slightly hydrolyzed. There was almost no hydrolysis of cephalexin or cephalosporin C. We found the isoelectric points for the major bands to be either 5.4 (two isolates) or 5.5 (two isolates).

Plasmid characterization and auxotypes of PPNG strains. The plasmid content and auxotype of all PPNG isolates submitted to our laboratory for molecular confirmation are shown in Table 3. (All plasmid studies were performed by extracting DNA as described above and subjecting it to agarose gel electrophoresis.) Agarose gel data is not presented since it confirms several previous studies (10, 28, 31). All strains contained a cryptic 2.6-megadalton plasmid. In addition, the majority (65.2%) of isolates carried both a 4.5-megadalton β-lactamase plasmid and a 24.5-megadalton transfer plasmid. The percentage of strains having only the 4.5- or 3.2-megadalton β-lactamase plasmids was 21.7 and 13.9%, respectively. Transformation of *N. gonorrhoeae* plasmid DNA into *E. coli* 490 confirmed that the β-lactamase genes were carried by the 4.5- and 3.2-megadalton plasmids.

TABLE 3. *Plasmid content and auxotype of PPNG strains*

Auxotype[a]	No. of isolates with plasmids of size (megadaltons)[b]:			Total no.	%
	4.5	4.5 + 24.5	3.2		
NR	3	20	6	29	42.0
PRO	12	25	2	39	56.5
ORN			1	1	1.5
Total no.	15	45	9	69	
%	21.7	65.2	13.0		100

[a] NR, Nonrequiring or prototrophic; PRO, proline requiring; ORN, ornithine requiring.
[b] All strains harbored the 2.6-megadalton cryptic plasmid.

Most of the PPNG isolates were characterized auxanographically as either proline-requiring or nonrequiring (Table 3). The single isolate which required ornithine for growth carried a 3.2-megadalton β-lactamase plasmid. Sixty-seven percent (6/9) of the strains with 3.2-megadalton plasmids were typed as being prototrophic. By contrast, only 38% (23/60) of the isolates harboring the 4.5-megadalton penicillinase plasmid were prototrophic, and the rest of these strains were proline requiring. It is interesting that virtually all of the PPNG strains were clustered in two auxotype groups. By comparison, data from a recent Canadian survey (Dillon, unpublished data) of 963 non-PPNG strains indicated that the nonrequiring auxotype was most prevalent (25.6%) but that proline-requiring strains comprised the fifth most prevalent auxotype group (9.4%). It is tempting to speculate that auxotype may play a role in determining plasmid stability.

Stability of β-lactamase expression in *N. gonorrhoeae* and *E. coli* transformants. One *N. gonorrhoeae* isolate harboring a 4.5-megadalton penicillinase-producing plasmid (GC1-5) and two isolates harboring 3.2-megadalton plasmids (GC1-14 and GC1-31), together with *E. coli* transformed with plasmid DNA from these strains (designated EC1-5, EC1-14, and EC1-31, respectively), were examined for the stability of their ampicillin resistance phenotypes under conditions of nonselective growth. As has been previously reported (31), the 4.5-megadalton penicillinase-producing plasmid was more stable in its *N. gonorrhoeae* host than the 3.2-megadalton plasmid. For example, after 5 days of nonselective subculture, 44% of GC1-5 colonies were ampicillin resistant as compared with 7.5 and 2.8% of GC1-14 and GC1-31 colonies.

The 3.2-megadalton plasmid was even more unstable in *E. coli* transformants with only 2.2 and 0.002% of the EC1-14 and EC1-31 transformants expressing ampicillin resistance on day 5 of subculture. In addition, by using a variety of methods, no plasmid DNA could be recovered from *E. coli* transformants harboring the 3.2-megadalton plasmid despite the phenotypic expression of penicillin resistance. By contrast, 100% of *E. coli* cells transformed with 4.5-megadalton plasmid DNA (EC1-5) were found to maintain ampicillin resistance even after 10 days of nonselective subculture. Plasmid DNA was isolated from these transformants.

DISCUSSION

The incidence of PPNG infections in Canada has increased appreciably during the past 2 years in contrast to the incidence for the previous 3 years of national surveillance. This increase mimics similar trends reported in The Netherlands (41) and in the United States (14). However, unlike these and other countries, Canada has not, as yet, developed an internal focus of PPNG infection. Most cases of PPNG in Canada were imported from areas where infections are endemic, for example, Southeast Asia and the west coast of the United States.

Although the incidence of PPNG infections in Canada is numerically insignificant when compared to the overall incidence of gonococcal infection in this country (5), it poses serious problems therapeutically. Our susceptibility studies confirm previous reports documenting the reduced sensitivity of these strains to both erythromycin and tetracycline (8, 28, 36) and their susceptibilities to spectinomycin, cefuroxime, and cefoxitin (40). Indeed, spectinomycin is the antibiotic recommended (6) for the treatment of uncomplicated infections due to PPNG.

The present study extends our initial report (8) and other subsequent reports (3, 15), pertaining to the activity of the macrolide antibiotic rosaramicin (42) against these strains. On a weight basis, rosaramicin proved to be the most potent of all the antibiotics tested.

In view of its considerable activity in inhibiting PPNG strains, and in view of the fact that rosaramicin can be present in higher concentrations in human prostatic, urethral, and vaginal secretions (2, 18) than its macrolide relative erythromycin, this antibiotic deserves greater clinical attention.

In discussing antimicrobial therapy for multiply resistant gonococci, Percival and Hart (26) recommended, among other antibiotics, spectinomycin, cefuroxime, cefoxitin, and chloramphenicol as possible therapeutic choices. The present study indicates that rosaramicin should be included as a viable alternative, pending clinical trials. Furthermore, chloramphenicol, because of its potentially toxic side effects, might be replaced by its more active (Table 1), less toxic analog (13), thiamphenicol.

The molecular characterization of the gonococcal plasmids from the PPNG isolates in this study is in agreement with observations by other workers (10, 28, 31). All isolates harbored the small 2.6-megadalton plasmid found in most gonococcal isolates (23, 33). In addition, two distinct sizes of penicillinase-producing plasmids were observed. The association of the 4.5-megadalton plasmid with isolates of Far Eastern origin and the 3.2-megadalton plasmid with isolates of African etiology has been reported in the literature (28, 32). In tracing the epidemiology of the Canadian isolates, not all strains could be contact traced to Far Eastern or African sources;

however, in those instances where field work established direct connections to other localities (4, 29), geographical etiologies of infection based on plasmid size have been confirmed. Our observations are in agreement with Roberts et al. (31) in that over 50% of the isolates carrying the 4.5-megadalton penicillinase plasmid also carried the 24.5-megadalton plasmid which has been characterized as a conjugative plasmid (9, 20, 37).

Reports concerning the association of auxotype and the size of the penicillinase plasmid (28, 31, 35) have indicated that *N. gonorrhoeae* strains carrying the 4.5-megadalton penicillinase plasmid are either prototrophic or proline dependent, whereas isolates of West African etiology, which carry the 3.2-megadalton plasmid, are arginine dependent. In the present study, both plasmid species were found only in prototrophic or in proline-requiring strains (only one isolate was arginine requiring). The prevalence of β-lactamase plasmids in these two auxotypes may suggest some hitherto unidentified plasmid stability mechanism.

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ADDENDUM IN PROOF

After this paper was accepted, we were informed that rosaramicin has been withdrawn from clinical investigation.

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