AUXOTYPES OF *NEISSERIA GONORRHOEAE* IN TORONTO: RELATIONSHIP TO PENICILLIN RESISTANCE AND TO SEX OF PATIENT

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SUMMARY. The relationship of auxotype to penicillinase production, penicillin susceptibility, and sex of the patient, has been studied in 8446 non-penicillinase-producing and 153 penicillinase-producing *Neisseria gonorrhoeae* (PPNG) strains isolated during 1980-1983 in Toronto. Proline-requiring types constituted 51% of the PPNG and 13% of the non-PPNG strains; ornithine-requiring types constituted only 4% of the PPNG compared with 12% of the non-PPNG isolates. The various auxotypes of the non-PPNG isolates showed marked differences in their degree of resistance to penicillin; and the proportions of each auxotype from male and female patients were dissimilar.

INTRODUCTION

Changes in the nutritional requirements of *Neisseria gonorrhoeae* were reported by Carifo and Catlin (1973); and the unique requirements of some penicillin-susceptible strains were described by Knapp and Holmes (1975). The distribution of different auxotypes in parts of Canada has been reported from Hamilton, Ontario (Hendry and Stewart, 1979; Stewart and Hendry, 1979) and from Montreal, Quebec (Turgeon and Granger, 1980).

Thompson *et al.* (1978), in the USA, studied the relation between auxotype and the degree of resistance to antibacterial drugs. Pauze and Dillon (1982) recorded a steadily increasing proportion of penicillinase-producing *N. gonorrhoeae* (PPNG) among their gonococcal isolates. Shtibel (1982) reported the increasing incidence of penicillin-resistant non-PPNG cultures and their degree of resistance.

The purpose of this study was to survey the prevalence of different auxotypes of *N. gonorrhoeae* in Toronto, and to study their relationship to penicillinase-production, penicillin-susceptibility, and the sex of the patients from whom they were isolated.

MATERIALS AND METHODS

Cultures. During 1980-1983, 8599 isolates from anogenital and pharyngeal infections were confirmed in our laboratory as *N. gonorrhoeae* by the following criteria: Gram-negative diplococci; oxidase positive; production of acid from dextrose (but not maltose or saccharose with Cystine Trypticase Agar sugars (BBL); no growth on nutrient agar (Difco) at 22°C;

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negative in *o*-nitrophenyl- β -D-galactopyranoside broth (Gibco); positive in the Difco Bacto-FA *N. gonorrhoeae* direct fluorescent antibody technique.

Penicillinase production was determined by the modified iodometric technique of Jorgensen *et al.* (1977). A good quality white bond starch paper was cut into square pieces 4 × 4 cm. These were soaked for 1 min in a solution of penicillin G (5 g/L), freshly prepared in monopotassium phosphate-sodium hydroxide buffer 0.05 M, pH 7.0. The squares were used immediately or stored frozen in polyethylene bags for up to 6 months.

A square was placed in the base of a 9-cm plastic petri dish, and inoculated with the test cultures, 1 cm apart, using a 2-mm platinum loop. A sheet of tissue paper was moistened with water and placed at the periphery of the petri dish to maintain a high level of humidity. The lid was replaced and the dish incubated, base down, for 15 min at 36°C. The base of the dish, with the test paper still adherent, was then inverted over a jar of iodine crystals for a few seconds. Penicillinase production was indicated by a white halo around the inoculated area; with a negative result, the area around the inoculum was blue.

Penicillin susceptibility. The test medium consisted of GC Base (Difco) enriched with Isovitalex 0.1% and lysed horse blood 1.5% (Shtibel, 1975). A plate dilution method was used, with penicillin concentrations of 0.12, 0.3, 0.6, 1.0, 2.0, 3.0 and 6.0 mg/L. (For inoculation and incubation, see below—*Auxotype determination.*)

Auxotyping medium. A chemically defined medium was modified from that of Catlin (1973); the ingredients are shown in Table I.

The base solution (part 1) was divided into 83-ml portions. Amino-acids, vitamins and growth factors (part 2) were added to each portion, one or more of the amino-acids being omitted from some portions to test for dependency (see below). The medium was adjusted to pH 7.2 and sterilised by passage through a 0.22- μ m membrane filter (Millipore). Finally, the molten agar base (part 3) was added to each portion and the medium was poured into 100 × 100 × 15 mm square petri dishes (Falcon) 30 ml/plate, and stored at 4°C.

Auxotype determination. For each test, seven media were used: a positive control plate consisted of the complete auxotyping medium; a negative control plate lacked cystine and cysteine (usual growth requirements of *N. gonorrhoeae*); the other five plates lacked either proline, citrulline (testing the ability of ornithine present in the medium to replace a citrulline requirement), citrulline and ornithine (precursor amino acids in arginine synthesis), uracil, or hypoxanthine.

Bacteria were suspended in a buffered salt solution to the opacity of MacFarland nephelometric standard no. 1, prepared by the addition of 0.1 ml of 1% barium chloride to 9.9 ml of 1% sulphuric acid. With a replicator, auxotyping plates were inoculated first, then the penicillin-susceptibility plates. Minimal inhibitory concentration (MIC) and nutritional requirements were determined after 48 h at 36°C in CO₂ 5%.

RESULTS

Of the 8599 isolates of *N. gonorrhoeae*, 153 were PPNG and 8446 were non-PPNG. Table II shows the relationship between penicillinase production and auxotype. Most of the PPNG isolates were either prototrophic (not requiring any of the amino-acids when each was omitted singly from the complete auxotyping medium) or proline requiring. The non-PPNG cultures had a wider range of nutritional requirements; in 2% of them, an unidentified requirement was revealed—they failed to grow on the complete auxotyping medium, although they grew well on the penicillin-susceptibility control medium.

Table III presents the distribution of penicillin susceptibility among the various auxotypes of non-PPNG. Of the 38% that were penicillin resistant (MIC $\geq$ 0.6 mg/L), most were prototrophic or proline requiring. Moderate resistance (MIC 0.3 mg/L) was found in 21%, and most of these were proline-citrulline-uracil requiring. The

TABLE I
Chemically defined medium

Stock solutions	Amount incorporated in one batch of medium	Stock solutions	Amount added to 83 ml of base solution
Part 1. Base solution.		Part 2. Amino-acids, vitamins and growth factors (Sigma)	
Salts solution (stored at 4°C)	288 ml	Cystine/cysteine solution (prepared fresh)	2 ml
NaCl (Fisher)	58.44 g	L-Cystine	0.36 g
K ₂ SO ₄ (Fisher)	8.71 g	L-Cysteine	0.42 g
MgCl ₂ ·6H ₂ O (Fisher)	4.07 g	Purified H ₂ O	50 ml
NH ₄ Cl (Fisher)	2.14 g	Proline solution (stored at -70°C)	1 ml
EDTA (Sigma)	0.034 g	L-Proline	1.16 g
Purified H ₂ O	1000 ml	Purified H ₂ O	50 ml
Sodium acetate solution (stored at 4°C)	28.8 ml	Citrulline solution (stored at -70°C)	1 ml
Sodium acetate (Fisher)	34 g	L-Citrulline	2.6 g
Purified H ₂ O	100 ml	Purified H ₂ O	50 ml
Sodium bicarbonate solution (prepared fresh)	28.8 ml	1N HCl (to dissolve)	2 ml
Sodium bicarbonate (Baker)	8.4 g	Ornithine solution (stored at -70°C)	1 ml
Purified H ₂ O	100 ml	L-Ornithine	1.98 g
Glucose (Difco)	14.4 g	Purified H ₂ O	50 ml
Glycerol lactate solution (stored at 4°C)	14.4 ml	Isoleucine solution (stored at -70°C)	1 ml
Glycerol (Oxford, Canada)	7.5 ml	L-Isoleucine	0.66 g
Lactic acid (Sigma)	7.5 ml	Purified H ₂ O	50 ml
Purified H ₂ O	50 ml	1N HCl (to dissolve)	2 ml
Purified H ₂ O	190.08 ml	Serine solution (stored at -70)	1 ml
KH ₂ PO ₄ (BDH)	7.2 g	L-Serine	2.1 g
K ₂ HPO ₄ (BDH)	10.8 g	Purified H ₂ O	50 ml
Oxaloacetic acid (Sigma)	0.090 g	1N HCl (to dissolve)	1 ml
Alkaline solution (prepared fresh)	14.4 ml	Methionine solution (stored at -70°C)	1 ml
Choline chloride (Sigma)	0.035 g	L-Methionine	0.75 g
Glutathione (Sigma)	0.77 g	Purified H ₂ O	50 ml
Spermine (Sigma)	1 g	1N HCl (to dissolve)	1 ml
Purified H ₂ O	50 ml	Valine solution (stored at -70°C)	1 ml
Glutamine solution (stored at -70°C)	7.2 ml	L-Valine	1.2 g
Glutamine (Sigma)	1.46 g	Purified H ₂ O	50 ml
Purified H ₂ O	50 ml	Leucine solution (stored at -70°C)	1 ml
Tryptophan solution (stored at -70°C)	14.4 ml	L-Leucine	1.96 g
Tryptophan (Sigma)	0.51 g	Purified H ₂ O	50 ml
Purified H ₂ O	50 ml	Histidine solution (stored at -70°C)	1 ml
Biotin solution (prepared fresh)	2.68 ml	L-Histidine	0.78 g
Biotin (Sigma)	8 g	Purified H ₂ O	50 ml
Ethanol 50%	10 ml	Threonine solution (stored at -70°C)	1 ml
CaCl ₂ solution (stored at -70°C)	2.68 ml	L-Threonine	1.2 g
CaCl ₂ ·2H ₂ O (Fisher)	1.85 g	Purified H ₂ O	50 ml
Purified H ₂ O	50 ml	(Warming to dissolve)	
Fe(NO ₃) ₃ solution (stored at 4°C)	5.76 ml	Lysine solution (stored at -70°C)	1 ml
Fe(NO ₃) ₃ ·9H ₂ O (Sigma)	0.2 g	L-Lysine	0.74 g
Purified H ₂ O	50 ml	Purified H ₂ O	50 ml
		Phenylalanine solution (stored at -70°C)	1 ml
		L-Phenylalanine	0.83 g
		Purified H ₂ O	50 ml
		Uracil solution (stored at -70°C)	1 ml
		Uracil	0.224 g
		1N NaOH	5 ml
		Purified H ₂ O	45 ml

contd over

TABLE I (contd)

		Stock solutions	Amount added to 83 ml of base solution
Part 3. <i>Agar base</i> .		Part 2 (contd)	
Purified agar*	5.2 g	Hypoxanthine solution (stored at -70°C)	1 ml
Purified H ₂ O	300 ml	Hypoxanthine	0.136 g
		1N NaOH	5 ml
		Purified H ₂ O	45 ml
		Vitamin solution (prepared fresh)	1 ml
		NADH	0.074 g
		Thiamine HCl	0.071 g
		Thiamine pyrophosphate	0.023 g
		Calcium pantothenate	0.048 g
		Inositol	0.011 g
		Purified H ₂ O	10 ml

* To remove fatty acids, 100 g of Oxoid Agar No.1 were treated successively with 500 ml of chloroform (BDH), 500 ml of methanol (BDH), and 450 ml of purified H₂O. The extracted agar was then dried before being weighed.

Purified H₂O had been distilled once, de-ionised once, and passed through an organic adsorptive cartridge and a 0.45- μ m membrane filter (Millipore).

TABLE II

Relationship between auxotype and penicillinase production in 8599 isolates of N. gonorrhoeae

Auxotype	Number (%) of isolates of	
	PPNG	non-PPNG
Proto	67 (44)	2590 (31)
Pro ⁻	78 (51)	1136 (13)
Orn ⁻	6 (4)	1026 (12)
Pro ⁻ Cit ⁻ Ura ⁻	0 (0)	1699 (20)
Orn ⁻ Ura ⁻ Hyx ⁻	0 (0)	945 (11)
Cit ⁻ Ura ⁻ Hyx ⁻	0 (0)	215 (3)
Other	2 (1)	690 (8)
NG	0 (0)	145 (2)
Total	153 (100)	8446 (100)

PPNG = penicillinase-producing *N. gonorrhoeae*; Proto = prototrophic (not requiring any of the amino-acids when each was omitted singly from the defined medium); Pro⁻ = proline requiring; Orn⁻ = ornithine requiring; Pro⁻Cit⁻Ura⁻ = proline-citrulline-uracil requiring; Orn⁻Ura⁻Hyx⁻ = ornithine-uracil-hypoxanthine requiring; Cit⁻Ura⁻Hyx⁻ = citrulline-uracil-hypoxanthine requiring; Other = cultures with nutritional requirements other than those listed above; NG = no growth on the complete auxotyping medium.

remaining 42% were susceptible to penicillin (MIC $\leq$ 0.12 mg/L), and had a wide range of nutritional requirements.

The isolates of non-PPNG from male and female patients had similar ranges of nutritional requirements (table IV) but the proportion of each auxotype was different for males and females. For example, prototrophes and proline-requiring strains were more frequent amongst the isolates from males (40% and 23%, respectively) than from females (22% and 9%). Conversely, proline-citrulline-uracil requiring strains, and ornithine-uracil-hypoxanthine requiring strains, were more frequent from females (31% and 24%) than from males (18% and 9%).

TABLE III

Relationship between auxotype of N. gonorrhoeae and in vitro susceptibility to penicillin, for 8446 isolates of non-PPNG

Auxotype	Number (%) of isolates for which the MIC of penicillin was		
	≤0.12 mg/L (susceptible)	0.3 mg/L (moderately resistant)	≥0.6 mg/L (resistant)
Proto	398 (5)	328 (4)	1864 (22)
Pro ⁻	194 (2)	41 (0.5)	901 (11)
Orn ⁻	965 (11)	61 (0.7)	0 (0)
Pro ⁻ Cit ⁻ Ura ⁻	0 (0)	1301 (15)	398 (5)
Orn ⁻ Ura ⁻ Hyx ⁻	945 (11)	0 (0)	0 (0)
Cit ⁻ Ura ⁻ Hyx ⁻	215 (3)	0 (0)	0 (0)
Other	648 (8)	25 (0.3)	17 (0.2)
NG	145 (2)	0 (0)	0 (0)
Total	3510 (42)	1756 (21)	3180 (38)

For abbreviations, see footnote to table II.

TABLE IV

Relationship between auxotype of N. gonorrhoeae and patient's sex, for strains of non-PPNG isolated in 1980-1981

Auxotype	Number (%) of isolates from	
	males (n = 1200)	females (n = 1200)
Proto	474 (40)	262 (22)
Pro ⁻	271 (23)	106 (9)
Orn ⁻	99 (8)	106 (9)
Pro ⁻ Cit ⁻ Ura ⁻	212 (18)	371 (31)
Orn ⁻ Ura ⁻ Hyx ⁻	102 (9)	287 (24)
Cit ⁻ Ura ⁻ Hyx ⁻	42 (4)	68 (6)

For abbreviations, see footnote to table II.

DISCUSSION

Of the 8599 cultures of *N. gonorrhoeae* tested in this study, 8454 required cystine or cysteine for growth, a finding consistent with that of Short *et al.* (1977). The remaining 145 failed to grow on the complete auxotyping medium and could not be evaluated for cystine and cysteine requirement.

Comparison between three Canadian studies of gonococcal auxotype—in Toronto (present study), in nearby Hamilton (Stewart and Hendry, 1979), and in more distant Montreal (Turgeon and Granger, 1980)—shows no obvious influence of geographical location. Prototrophes constituted 31% of non-PPNG isolates in Toronto (table II), only 13% in Hamilton, but 33% in Montreal. The proportions of auxotypes requiring ornithine-uracil-hypoxanthine and citrulline-uracil-hypoxanthine varied more widely: 14% in Toronto, 28% in Hamilton and 44% in Montreal. Isolates requiring only proline or ornithine accounted, respectively, for 13% and 12% in Toronto, 10% and 5% in Hamilton and 16% and 4% in Montreal. Strains requiring proline-citrulline-uracil

formed 20% in Toronto and 39% in Hamilton, but were not mentioned in the Montreal study.

There are noticeable differences in the degree of penicillin resistance between the Toronto auxotypes (table III) and those reported from Hamilton. In Toronto, prototrophic and proline-requiring cultures showed a broad distribution of MICs, ranging from ≤ 0.12 mg/L to ≥ 0.6 mg/L, whereas, in the Montreal study, the highest MICs were 0.08 mg/L for prototrophes and 0.32 mg/L for the proline-requiring strains. For ornithine-requiring cultures, the MICs of the Toronto isolates varied from ≤ 0.12 mg/L to 0.3 mg/L, whereas the peak was 0.08 mg/L in the Hamilton study. For strains requiring proline-citrulline-uracil, in Toronto the MICs were $0.3\text{--}\geq 0.6$ mg/L, but in Hamilton they were much lower (≥ 0.08 mg/L). Therefore, the present results demonstrate a higher degree of penicillin resistance for all the auxotypes isolated in Toronto.

Although the PPNG cultures in the present study constituted only 2% of the 8599 isolates, they accounted for 23% of the total PPNG in Canada (Pauze and Dillon, 1982). Thus, along with 22% from other laboratories in Ontario, the Province of Ontario provided 45% of the Canadian total. Of the PPNG isolates in Toronto (table II), 44% were prototrophic, 51% proline-requiring, and 4% ornithine-requiring. Similar results were reported by Pauze and Dillon—50% prototrophic, 49% proline-requiring, and only 1% ornithine-requiring.

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