

Reduced Susceptibility to Azithromycin and High Percentages of Penicillin and Tetracycline Resistance in *Neisseria gonorrhoeae* Isolates from Manaus, Brazil, 1998

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Background: The identification of *Neisseria gonorrhoeae* isolates resistant to antimicrobial agents currently recommended for the treatment of gonococcal infections continues to escalate globally. Thus, in some areas, resistance to fluoroquinolone drugs is commonplace; several reports document resistance to third-generation cephalosporins, and the sporadic isolation of spectinomycin-resistant isolates continues unabated. Gonococcal resistance to azithromycin, an antibiotic used for the primary treatment of gonococcal infections in some Latin American countries, also has been described. Because the prevalence of resistant isolates is insufficiently documented in many areas of Latin America, the efficacy of locally recommended therapies for gonococcal infections is often unknown.

Goal: To determine the antimicrobial susceptibility and strain types of *N gonorrhoeae* isolates collected in Manaus, Brazil. These data will establish antimicrobial susceptibility baseline data for the region as a reference point for future surveillance.

Study Design: Consecutive *N gonorrhoeae* isolates from urethral and endocervical specimens were collected and examined for identity, antimicrobial susceptibility, and strain type (plasmid content, *tetM* type, auxotype, and serovar).

Results: Most of the isolates (65/81; 85.2%) were resistant to tetracycline, penicillin, or both, with the majority (n = 62) carrying plasmid-mediated resistance to tetracycline (tetracycline-resistant *N gonorrhoeae* [TRNG]). All of the TRNG contained the Dutch-type *tetM* plasmid, and 18 were A/S class NR/IA-02. Penicillinase-producing *N gonorrhoeae* comprised 8.2% (7/81) of the isolates. Of these seven isolates, four also

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were TRNG, and two carried chromosomal resistance to tetracycline. The isolates were susceptible to ciprofloxacin, spectinomycin, and ceftriaxone. However, 23 isolates were characterized by reduced susceptibility to azithromycin (MIC, 0.25–0.5 µg/ml), and one isolate had reduced susceptibility to ciprofloxacin (MIC, 0.25 µg/ml).

Conclusions: This study supports the continued use of third-generation cephalosporins, spectinomycin, and fluoroquinolone drugs for the primary treatment of gonococcal infections in Manaus. The occurrence of isolates with reduced susceptibility to azithromycin and ciprofloxacin underscores the importance of ongoing antimicrobial susceptibility monitoring to support decisions regarding appropriate drugs for the treatment of gonococcal infections.

INFECTIONS CAUSED BY *Neisseria gonorrhoeae* are curable provided effective antimicrobial agents are used for treatment.^{1,2} Penicillin and tetracycline have not been recommended for the treatment of gonococcal infections for more than a decade in most countries because of widespread resistance to these agents.² However, gonococcal isolates resistant to “effective” agents, especially fluoroquinolone drugs, have been reported from many countries,^{3–8} and the steady development of chromosomally mediated resistance limits the possibility of their continued use.^{9–11} Thus, determining the prevalence of resistant isolates to ascertain continued treatment efficacy is fundamental for the success of public health programs to control sexually transmissible diseases.

Antimicrobial resistance in *N gonorrhoeae* can be either plasmid or chromosomally mediated, and isolates often

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carry a combination of resistance phenotypes and genotypes to different antimicrobial agents.^{3,4} The most common type of gonococcal resistance worldwide is caused by penicillinase-producing *N gonorrhoeae* (PPNG) isolates.^{3,12,13} Strains with high-level plasmid-mediated tetracycline resistance encode a *tetM* determinant carried on two classes of 25.2-MDa conjugative gonococcal plasmid: Dutch type and American type.^{14–16} Chromosomally mediated resistance to a wide spectrum of antimicrobial agents, including fluoroquinolone and macrolides, occurs through a number of mutations in different genes, and the simultaneous effects of different mutations may be additive.^{7,10,11,17}

Previous studies in Brazil have recorded the presence of both penicillin- and tetracycline-resistant isolates. The first reported cases of PPNG included the cities of Recife in 1983,¹⁸ São Paulo in 1985,¹⁹ and Rio de Janeiro in 1985.²⁰ During the 1980s, chromosomal resistance to tetracycline was reported from cities such as Bello Horizonte (5.5% of isolates)²¹ and Florianópolis (69.1% of isolates).²² High percentages (54.3%) of isolates with chromosomal resistance to penicillin also have been noted in Florianópolis.²² Despite several excellent studies documenting the susceptibility of gonococcal isolates to antimicrobial drugs in Brazil, these surveys have tended to be sporadic, and in many regions of the country, baseline data have not yet been published in the international literature.

The current study was part of the broader Gonococcal Antimicrobial Surveillance Program (GASP) in Latin America and the Caribbean. The objective of the study was to develop baseline data on the antimicrobial susceptibility of *N gonorrhoeae* isolates from Manaus, a fluvial port in the Brazilian state of Amazonas. Several classes of resistant isolates are described, including isolates with reduced susceptibility to azithromycin, an antibiotic recommended in Brazil both for the primary treatment of gonococcal infections and for the simultaneous treatment of chlamydial infections.

Methods

Bacterial Strains and Growth Conditions

Neisseria gonorrhoeae consecutive isolates were collected at the Fundação Alfredo da Matta STD/AIDS clinic and laboratory in Manaus, Brazil, from May 1998 to September 1998. Patients from all parts of the city visited the clinic, with most (58.9%) cases self-referred. During this 5-month period, 247 specimens from either urethral (71.2%) or cervical (28.8%) discharges were collected, 168 of which were positive for *N gonorrhoeae* (29 from women and 139 from men).

The specimens were inoculated directly onto modified Thayer Martin, medium and the plates were placed in jars to which a tablet of sodium carbonate was placed on water-soaked gauze, thereby generating an atmosphere of about

7% carbon dioxide (CO₂). These jars were incubated at 35 °C for 24 to 48 hours. The isolates were presumptively identified as *N gonorrhoeae* by Gram stain, oxidase, and catalase tests.²³ Confirmation of identity, after subculture on chocolate agar, was based on carbohydrate use tests using cystine trypticase agar medium (CTA) supplemented with 1% sugars prepared locally.²³ Gonococcal isolates were stored at –70 °C in brain heart infusion broth (Difco Laboratories, Detroit, MI) containing 20% glycerol. Frozen specimens were subsequently shipped on dry ice to the Coordinating Center for GASP in the Americas and the Caribbean, at Ottawa, via the National Program on STD/AIDS, Ministry of Health, Brazil. Of 120 strains (the number viable from the original sample), 81 were sent from Manaus to Ottawa. Their identity was reconfirmed in Ottawa using standard methods.²³

Antimicrobial Susceptibility Testing

Before their antimicrobial susceptibility was tested, gonococcal isolates were subcultured on a GC medium base (GCMB; Difco), to which modified Kellogg's supplements were added, and incubated 18 to 24 hours at 35 °C in a humid atmosphere with 5% CO₂. The MICs of the isolates were determined by the agar dilution method for penicillin (Wyeth-Ayerst Canada Inc., St. Laurent, QC), tetracycline (Pfizer Canada Inc., Pte. Claire, QC), spectinomycin (Upjohn Canada, Don Mills, ON), ceftriaxone (Hoffman-La Roche, Mississauga, ON), ciprofloxacin (Bayer Inc., Etobicoke, ON), and azithromycin (Pfizer).²⁴ Control strains have been described previously.²⁵ The interpretive criteria were those recommended by the National Committee for Clinical Laboratory Standards (NCCLS).²⁴

The isolates were presumptively defined as PPNG if they were β -lactamase positive, as determined by testing with nitrocefin. Tetracycline-resistant *Neisseria gonorrhoeae* (TRNG) were identified initially on the basis of MICs ≥ 16 $\mu\text{g/ml}$ to tetracycline. Isolates characterized as both PPNG and TRNG (PP/TRNG) were classified as being β -lactamase positive and having MICs ≥ 16 $\mu\text{g/ml}$ to tetracycline. Chromosomal resistance to tetracycline was defined as an MIC of 2 to 8 $\mu\text{g/ml}$. Chromosomally mediated resistance to penicillin was defined as an MIC of ≥ 2 $\mu\text{g/ml}$ in non-PPNG isolates. On the basis of definitions used in the literature, isolates were considered to be resistant to azithromycin if their MICs were ≥ 2 $\mu\text{g/ml}$, and they were classified as having reduced susceptibility if their MICs were 0.25 to 1.0 $\mu\text{g/ml}$.^{11,25,26} Isolates classified as resistant to ciprofloxacin exhibited MICs ≥ 1 $\mu\text{g/ml}$ or more, and those resistant to spectinomycin had MICs ≥ 128 mg/ml.²⁴ Because breakpoints have not been described for third-generation cephalosporins, isolates with MICs ≤ 0.25 $\mu\text{g/ml}$ were considered to be susceptible to ceftriaxone.²⁴

TABLE 1. Strain Types and Antibiotic Resistance Types of *Neisseria gonorrhoeae* Isolates From Manaus, Brazil

A/S	Isolates						
	PPNG n (%)	PP/TRNG n (%)	TRNG n (%)	PPNG + CMTR n (%)	CMTR n (%)	Susceptible n (%)	Total n (%)
NR/IA-2			18 (22.2)			1 (1.2)	19 (23.5)
NR/IB-3		3 (3.7)	7 (8.6)		1 (1.2)	4 (4.9)	15 (18.4)
NR/IB-8			7 (8.6)				7 (8.6)
P/IB-1			6 (7.4)				6 (7.4)
NR/IA-6			4 (4.9)				4 (4.9)
NR/IB-1			3 (3.7)				3 (3.7)
NR*			3 (3.7)				3 (3.7)
P/IA-8			2 (2.5)				2 (2.5)
NR/IA-14	1 (1.2)		2 (2.5)				3 (3.7)
NR/IB-32			2 (2.5)				2 (2.5)
P/IA-2			1 (1.2)			1 (1.2)	2 (2.5)
NR/IB-5			1 (1.2)		2 (2.5)		3 (3.7)
NR/IB-6		1 (1.2)	1 (1.2)				2 (2.5)
NR†			1 (1.2)				1 (1.2)
NR/IA-21						2 (2.5)	2 (2.5)
P/IB-8						2 (2.5)	2 (2.5)
H/IB-1						1 (1.2)	1 (1.2)
NR/IB-14						1 (1.2)	1 (1.2)
NR/IB-22				2 (2.5)	1 (1.2)		3 (3.7)
Total	1 (1.2)	4 (4.9)	58 (71.6)	2 (2.5)	4 (4.9)	12 (14.8)	81 (100)

*TRNG strains reacted to both IA and IB epitopes.

†TRNG strain not tested for serotype.

PPNG = penicillinase-producing *N gonorrhoeae*; TRNG = tetracycline-resistant *N gonorrhoeae*; PP/TRNG = PPNG and TRNG; CMTR = chromosomally mediated tetracycline resistance; NR = nonrequiring or wild type; P = proline-requiring; H = hypoxanthine-requiring.

Typing Isolates by Plasmid Content, Auxotype, Serovar, and tetM Content

The plasmid content, auxotypes, and serovars of the isolates were determined as described previously.^{12,27,28} Reagents for serovar determination were provided by Syva (Palo Alto, CA). The *tetM* gene was typed using a method modified from Xia et al.¹⁵

Results

The susceptibility to penicillin, tetracycline, spectinomycin, ceftriaxone, ciprofloxacin, and azithromycin was ascertained for 81 isolates of *N gonorrhoeae*. As shown in Table 1, 69 isolates (85.2%) were resistant to tetracycline, penicillin, or both, with 68 resistant to tetracycline (62 TRNG and 6 chromosomally resistant to tetracycline [CMTR]) and 7 resistant to penicillin (8.6%). One TRNG isolate also manifested reduced susceptibility to ciprofloxacin (MIC, 0.25 $\mu\text{g/ml}$; Table 2). All penicillin-resistant isolates were PPNG, with four PP/TRNG and two PPNG also displaying chromosomal resistance to tetracycline (PPNG + CMTR; Table 1). The two PPNG/CMTR isolates also exhibited reduced susceptibility to azithromycin (MIC, 0.5 $\mu\text{g/ml}$).

Isolates were susceptible to spectinomycin, ciprofloxacin, and ceftriaxone (Table 2). However, reduced susceptibility to azithromycin was noted in 23 isolates (28.4%, Table 2), with 19 isolates having MICs of 0.25 $\mu\text{g/ml}$ and 4 isolates with MICs of 0.5 $\mu\text{g/ml}$.

Typing by Plasmid Content, Auxotype, and Serovar

All TRNG isolates ($n = 62$) contained 25.2-MDa plasmids, which were of the Dutch type. Of the seven PPNG isolates, three carried the 3.05-MDa Toronto-type β -lactamase plasmid (also TRNG isolates), one carried the 3.2-MDa Africa-type β -lactamase plasmid (also a TRNG isolate), and three contained the 3.2-MDa Africa-type β -lactamase plasmid as well as a 24.5-MDa transfer plasmid (two classified as CMTR and one as susceptible to tetracycline).

The isolates tested were predominantly of the nonrequiring (NR) auxotype (68 isolates; 84%; Table 1), with 12 other isolates requiring proline and 1 requiring histidine (Table 1). The predominant serovars were IA-2 (21 isolates;

TABLE 2. Susceptibility of *Neisseria gonorrhoeae* Isolates From Manaus to Spectinomycin, Ceftriaxone, Ciprofloxacin, and Azithromycin

Antimicrobial Agent	MIC Range ($\mu\text{g/ml}$)	MIC ₅₀ ($\mu\text{g/ml}$)	MIC ₉₀ ($\mu\text{g/ml}$)
Spectinomycin	8.0–16.0	16.0	16.0
Ceftriaxone	0.00025–0.016	0.004	0.008
Ciprofloxacin	0.004–0.25	0.004	0.008*
Azithromycin	0.032–0.5	0.125	0.25†

*All isolates inhibited by 0.016 $\mu\text{g/ml}$ except one tetracycline-resistant *Neisseria gonorrhoeae* (TRNG) isolate with an MIC of 0.25 $\mu\text{g/ml}$.

†A total of 23 isolates had reduced susceptibility (MIC, 0.25–0.5 $\mu\text{g/ml}$) to azithromycin.

25.9%; Table 1), IB-3 (15 isolates; 18.5%), IB-1 (10 isolates; 12.3%), and IB-8 (9 isolates; 8.6%). More than 50% of the isolates belonged to A/S classes NR/IA-2 (23.5%), NR/IB-3 (18.4%), and NR/IB-8 (8.6%), although 18 different A/S classes were identified altogether (Table 1). Three strains possessed both 1A and 1B epitopes (Table 1).

The TRNG isolates could be grouped into 14 different A/S classes. Eighteen (29%) belonged to A/S class NR/IA-2 (Table 1), and were all susceptible to penicillin (MIC, 0.25 $\mu\text{g/ml}$), spectinomycin (MIC, 16 $\mu\text{g/ml}$), ceftriaxone (MIC, 0.004 $\mu\text{g/ml}$), ciprofloxacin (MIC, 0.004 $\mu\text{g/ml}$), and azithromycin (MIC, 0.25 $\mu\text{g/ml}$). The PPNG isolates belonged to four different A/S classes: Three were NR/IB-3 as well TRNG, with two containing the Toronto-type plasmid and one the Africa-type β -lactamase-producing plasmid; two were NR/IB-22 and contained Africa-type plasmids; and one each were NR/IB-06 (TRNG, Toronto-type plasmid) and NR/IA-14 (Africa-type plasmid). All the isolates carried the gonococcal cryptic plasmid (2.6 Mda), and all the non-TRNG isolates also carried the 24.5-Mda transfer plasmid.

The 23 isolates with reduced susceptibility to azithromycin belonged to nine different A/S classes: Eight were NR/IA-2 and also TRNG; three each were NR/IB-5 (1 TRNG and 2 CMTR) and NR/IB-22 (all CMTR and 2 PPNG); two each were NR/IB-3 (tetracycline MIC, 1 $\mu\text{g/ml}$), P/IA-8 (TRNG), and P/IB-1 (TRNG); and single isolates were classified as NR/IB-14 (PPNG; tetracycline MIC, 1 $\mu\text{g/ml}$), NR/IA-14 (tetracycline MIC, 1 $\mu\text{g/ml}$) and P/IA-2 (TRNG).

Discussion

This current study was undertaken to develop baseline antimicrobial susceptibility data in Manaus, Brazil. It is part of a broader national Gonococcal Antimicrobial Surveillance Program (GASP) being initiated in Brazil, which is linked to the overall program in the Americas and the Caribbean. The long-term intent of the GASP programs is to have various regional centers perform their own testing so that results can be used to adopt appropriate national and regional treatment guidelines. At the same time, regional data provide an important basis for evaluating and comparing international trends in resistance and treatment efficacy.

Data from Manaus reflect the high percentages (84% overall; 76.5% TRNG) of tetracycline-resistant isolates (either chromosomal or, more recently, plasmid-mediated) noted in Latin America and the Caribbean for at least two decades. For example, 57% of the isolates tested in Cordoba, Argentina during 1983–1984 had tetracycline MICs indicative of chromosomally mediated resistance (i.e., 2–4 $\mu\text{g/ml}$).²⁹ A study in Pôrto Alegre, Brazil in 1984 (Dillon and Gervase, unpublished data) determined that 110 (44.5%) of 247 isolates tested carried chromosomal resis-

tance to tetracycline, with almost no cross-resistance to other agents such as penicillin or erythromycin. A more recent report from Uruguay indicated that 57.8% of gonococcal isolates exhibited CMTR,³⁰ whereas a report about a decade earlier from Mexico showed that 41% of the isolates displayed reduced susceptibility or resistance to tetracycline.³¹ High percentages of isolates with plasmid-mediated resistance to tetracycline have been noted in Nicaragua (22%),³² Jamaica (69%),³³ Guyana (87.1%),²⁵ St. Vincent (22%),²⁵ and Trinidad (15.3%).²⁵ By comparison, approximately 6% to 7% of the isolates tested in the United States between 1988 and 1998 were TRNG, and approximately 14% exhibited CMTR.³⁴ In Canada, TRNG increased from 2.3% in 1990 to approximately 20% of the isolates tested in 1995 (Ng and Dillon, unpublished data).

In the current study, all the TRNG isolates from Manaus were classified as the Dutch type. Most of the TRNG isolates typed from Latin America and Caribbean countries have been the Dutch type, with only sporadic reports of isolates with the American type of *tetM*-containing plasmid.^{30,35,36} Most of these TRNG isolates from Manaus belonged to A/S classes NR/IA-2, NR/IB-3, and NR/IB-8 and P/IB-1. These A/S classes differ from those previously reported for TRNG from Brazil, all of which were Dutch-type TRNG of the A/IA class.^{36,37} Other TRNG isolates from Latin America, while predominantly the Dutch type, have been typed into a number of different A/S classes, indicating that circulating TRNG isolates, as expected, are not clonal.^{30,36}

Early publications documented only sporadic isolations of PPNG from various parts of Brazil, and anecdotal reports implied that the prevalence of PPNG was low.^{18–21} However, a multicenter study in 1989 showed that 51% (32/63) of the gonococcal isolates tested were β -lactamase-producing.³⁸ Other countries in Latin America and the Caribbean report even higher percentages of penicillinase-producing isolates, for example, 60% in Honduras,³⁹ 78% in Nicaragua,³² 58.6% in Jamaica,³³ 30% to 50% in Barbados,⁴⁰ 54% in Uruguay,³⁰ and 72.8% in Guyana.²⁵ The percentage of PPNG (8.6%) reported in the current study, while lower, is still high enough to indicate that penicillin drugs should not be used to treat gonococcal infections. It has been suggested that when PPNG account for 1% to 3% of all *N gonorrhoeae* isolates, other drugs with proven efficacy should be chosen.⁴¹

Although the antimicrobial agents used for the treatment of gonorrhea in Brazil until 1997 included ampicillin, current treatment guidelines do not recommend this antibiotic.⁴² The primary drugs recommended are ciprofloxacin, ofloxacin (the drug of choice), azithromycin, ceftriaxone, and thiamphenicol.⁴² Penicillin was last used at the Fundação Alfredo da Matta Clinic in April 1997. At the time the isolates for the current study were collected, the clinic in Manaus used regimens of ciprofloxacin and thiamphenicol

for the primary treatment of gonorrhea coupled with azithromycin or doxycycline for concurrent chlamydial infections. However, it is possible that other centers in the city of Manaus and in the interior of the state of Amazonas still use penicillin or ampicillin for the treatment of gonorrhea.

Interestingly, 23 isolates from Manaus displayed decreased susceptibility to azithromycin. In a few countries, *N gonorrhoeae* strains either resistant or less susceptible to azithromycin have been reported, including some from South America.^{11,43,44} The development of resistance to erythromycin might in fact foreshadow resistance to azithromycin because cross-resistance between the two has been noted.⁴³ However, this "decreased" susceptibility should be studied further because the reduction in pH after incubation in 5% CO₂, an environment required for the growth of *N gonorrhoeae* isolates, can cause the ionization of macrolide molecules, slowing their entry into bacterial cells. This may result in an increase in the MIC of the antibiotic.⁴⁵

In conclusion, the current study represents one of the first studies to emerge from the developing national GASP program in Brazil. It presents a snapshot of resistance phenotypes during a 5-month period, and amply documents percentages of tetracycline and penicillin resistance high enough to contraindicate their use for the treatment of gonococcal infections. In addition, the detection of isolates with presumed reduced susceptibility to azithromycin and other currently recommended drugs, such as the fluoroquinolone, indicates that susceptibility to this drug should be closely monitored.

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