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MECHANISMS OF RESISTANCE

Equations To Predict Antimicrobial MICs in *Neisseria gonorrhoeae* Using Molecular Antimicrobial Resistance Determinants

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ABSTRACT The emergence of *Neisseria gonorrhoeae* strains that are resistant to azithromycin and extended-spectrum cephalosporins represents a public health threat, that of untreatable gonorrhea infections. Multivariate regression modeling was used to determine the contributions of molecular antimicrobial resistance determinants to the overall antimicrobial MICs for ceftriaxone, cefixime, azithromycin, tetracycline, ciprofloxacin, and penicillin. A training data set consisting of 1,280 *N. gonorrhoeae* strains was used to generate regression equations which were then applied to validation data sets of Canadian ($n = 1,095$) and international ($n = 431$) strains. The predicted MICs for extended-spectrum cephalosporins (ceftriaxone and cefixime) were fully explained by 5 amino acid substitutions in PenA, A311V, A501P/T/V, N513Y, A517G, and G543S; the presence of a disrupted *mtrR* promoter; and the PorB G120 and PonA L421P mutations. The correlation of predicted MICs within one doubling dilution to phenotypically determined MICs of the Canadian validation data set was 95.0% for ceftriaxone, 95.6% for cefixime, 91.4% for azithromycin, 98.2% for tetracycline, 90.4% for ciprofloxacin, and 92.3% for penicillin, with an overall sensitivity of 99.9% and specificity of 97.1%. The correlations of predicted MIC values to the phenotypically determined MICs were similar to those from phenotype MIC-only comparison studies. The ability to acquire detailed antimicrobial resistance information directly from molecular data will facilitate the transition to whole-genome sequencing analysis from phenotypic testing and can fill the surveillance gap in an era of increased reliance on nucleic acid assay testing (NAAT) diagnostics to better monitor the dynamics of *N. gonorrhoeae*.

KEYWORDS MIC, *Neisseria gonorrhoeae*, antimicrobial resistance, molecular analysis, whole-genome sequencing

Neisseria gonorrhoeae is a Gram-negative organism causing gonorrhea, the second most prevalent sexually transmitted bacterial infection in Canada after *Chlamydia trachomatis* (https://www.canada.ca/en/public-health/services/publications/drugs-health-products/national-surveillance-antimicrobial-susceptibilities-neisseria-gonorrhoeae-annual-summary-2017.html). Increasing *in vitro* antimicrobial resistance

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(AMR) in *N. gonorrhoeae* threatens the long-term sustainability of current dual-antimicrobial therapeutic regimens (ceftriaxone and azithromycin) and raises concerns for future treatment of gonorrhea (1–6).

The antimicrobial resistance mechanisms for *N. gonorrhoeae* have been extensively documented (7, 8) and with few exceptions can fully explain the observed antimicrobial phenotypes. Azithromycin resistance in *N. gonorrhoeae* has been attributed primarily to 23S rRNA point mutations (9, 10), the overexpression of the MtrCDE efflux pump mostly caused by disruptions in the promoter region of an *mtrR* repressor such as the −35A deletion, mosaic *N. meningitidis*-like and WHO-P-like disrupted sequences (11, 12), and smaller contributions from the MtrR A39T and G45D mutations (13, 14). Other less common macrolide resistance mechanisms include the *mef* and MacAB efflux pumps, the presence of *ermA*, *ermB*, *ermC,* and *ermF* genes encoding 23S rRNA methylases, and mutations in the ribosomal genes *rplD* and *rplV* (7). Penicillin and extended-spectrum cephalosporin resistance has been associated with mutations and recombination within *penA*, *porB*, *ponA,* and the presence of *bla* (8, 15–17). Fluoroquinolone resistance has been well described by variations in GyrA at amino acid positions S91 and D95 and in ParC at positions D86, S87, S88, and E91 (18–20), while tetracycline resistance has been attributed to the presence of *tetM* (21) and point mutations in *rpsJ*, *mtrR,* and *porB* (22).

Monitoring the dissemination and dynamics of antimicrobial-resistant *N. gonorrhoeae* has traditionally relied upon *in vitro* phenotypic susceptibility testing of bacterial cultures; however, the increase in nucleic acid assay testing (NAAT) to diagnose gonorrhea has resulted in fewer bacterial cultures being available for testing. Over 80% of gonococcal infections in Canada are now detected using NAAT, and some jurisdictions no longer maintain the capacity to culture the bacteria (https://www.canada.ca/en/public-health/services/publications/drugs-health-products/national-surveillance-antimicrobial-susceptibilities-neisseria-gonorrhoeae-annual-summary-2017.html) (23). This gap in antimicrobial susceptibility surveillance data may be addressed by the development of novel molecular-based techniques to determine antimicrobial resistance (24–26) by detecting the presence/absence of specific genes or single nucleotide variations (SNVs).

Multivariate regression modeling as a method to predict MICs was first introduced in 2016 to determine azithromycin MICs in *N. gonorrhoeae* (27) and later expanded in 2017 to include other antimicrobials (28). In this study, we employ a statistical approach not only to determine the categorical antimicrobial resistance or susceptibility, but also to determine the contribution of each molecular determinant to antimicrobial MIC values and provide simple mathematical equations that can be applied to determine the MICs for ceftriaxone, cefixime, azithromycin, penicillin, tetracycline, and ciprofloxacin.

RESULTS

Regression analysis indicated that the predicted MIC (MIC_{pred}) for cephalosporins depended upon 5 amino acid substitutions in PenA (A311V, A501P/T/V, N513Y, A517G, and G543S), the presence of a disrupted *mtrR* promoter (*N. meningitidis*-like or WHO-P-like), an amino acid change in PorB at G120, and the L41P substitution in PonA. The molecular determinants having the largest effect on ceftriaxone MICs were the A501P/T/V, A311V, N513Y, and the PorB G120 amino acid substitutions, producing an adjusted R^2 of 0.721 (see the supplemental material). All ceftriaxone MIC_{pred} values calculated from the regression equation (Fig. 1) agreed within one doubling dilution with the published MIC (MIC_{pub}) (29) and phenotypically determined MIC (MIC_{pheno}) values for the panel of 14 WHO reference strains (Table 1). There was 95.0% (1,040/1,095) concordance within one doubling dilution of the MIC_{pheno} of the Canadian validation strains (Table 2). There was only a single Canadian isolate with a ceftriaxone MIC_{pheno} of 0.5 mg/liter (corresponding to the resistant interpretative breakpoint) in the Canadian data set. Therefore, meaningful sensitivity and specificity values could not be

$$\textbf{MIC value (mg/L)} = 2^{(round[MIC\ Increment\ Regression\ Equation])}$$

$$\textbf{AZI MIC (mg/L)} = 2^{[(-2.89+(A2059G\times 2.33)+(C2611T\times 1.27)+(mtrRpM\times 3.47)+(mtrRpP\times 4.89)+(mtrRpA\times 0.80)+(ermB\times 2.38)+(ermC\times 3.0)+(A39T\times 0.41)+(G120\times 0.52)+(PonA\times 0.25)]}$$

$$\textbf{CIP MIC (mg/L)} = 2^{[(-7.62+(S91\times 5.67)+(D86\times 5.05)+(S87R\times 5.67)+(S87I\times 4.18)+(S87C\times 5.95)+(S87N\times 1.79)+(S88\times 1.45)+(E91\times 5.43))]}$$

$$\textbf{CFM MIC (mg/L)} = 2^{[(-7.21+(mtrRpMP\times 0.47)+(G120\times 0.75)+(PonA\times 0.63)+(A311V\times 4.17)+(A501P\times 4.92)+(A501T\times 1.67)+(A501V\times 1.72)+(N513Y\times 2.92)+(A517G\times 0.47)))]}$$

$$\textbf{CFX MIC (mg/L)} = 2^{[(-7.72+(mtrRpMP\times 0.54)+(G120\times 1.38)+(PonA\times 0.67)+(A311V\times 3.90)+(A501P\times 5.15)+(A501T\times 1.51)+(A501V\times 1.92)+(N513Y\times 1.53)+(A517G\times 0.43)+(G543S\times 0.48))]}$$

$$\textbf{PEN MIC (mg/L)} = 2^{[(-3.21+(bla\times 6.42)+(mtrRpMP\times 0.56)+(G120\times 1.34)+(PonA\times 1.55)+(N513Y\times 1.25)+(A517G\times 1.28)+(G543S\times 0.42))]}$$

$$\textbf{TET MIC (mg/L)} = 2^{[(-1.83+(mtrRpANY\times 0.62)+(A39T\times 0.26)+(G120\times 0.79)+(A121\times 0.22)+(rpsJ\times 2.11)+(tetM\times 4.15))]}$$

Where: ***A2059G*** and ***C2611T*** have values of 0 to 4 to indicate the number of 23s rRNA alleles with the respective mutation and a value of 1 or 0 represents the presence or absence, respectively for: ***mtrRpM*** (*N. meningitidis*-like *mtrR* promoter sequence); ***mtrRpP*** (WHO-P-like sequence); ***mtrRpA*** (-35A deletion); ***mtrRpMP*** (*N. meningitidis* -like or WHO-P-like *mtrR* promoter sequence); ***mtrRpANY*** (*N. meningitidis* -like or WHO-P-like *mtrR* promoter sequence or -35A deletion); ***bla***, ***ermB***; ***ermC, tetM*** (presence of gene); ***A39T*** (MtrR A39T); ***G120*** (any PorB G120 substitution); ***A121*** (any PorB A121 substitution); ***PonA*** (PonA L421P); ***S91*** (any GyrA S91 substitution); ***D86*** (any ParC S86 substitution); ***S97R***, ***S87I***, ***S87C*** and ***S87N*** (ParC amino acid substitutions); ***S88*** (any ParC S88 substitution); ***E91*** (any ParC E91 substitution); ***A311V***, ***A501P***, ***A501T***, ***A501V***, ***N513Y***, ***A517G*** and ***G543S*** (PenA amino acid substitutions); ***rpsJ*** (RpsJ V57M substitution).

FIG 1 Multivariate regression equations to determine the MIC values of azithromycin (AZI), ceftriaxone (CFX), cefixime (CFM), penicillin (PEN), ciprofloxacin (CIP), and tetracycline (TET) based on molecular resistance determinants of *Neisseria gonorrhoeae*.

calculated; however, there were no major (MA) or very major (VMA) interpretative errors.

The MIC_{pred} values for cefixime were similarly dependent upon the PenA mutations associated with those influencing ceftriaxone MICs, except for PenA G543S, which had an insignificant *P* value of 0.514 (see the supplemental material) and was removed from the regression model for cefixime MIC calculation. PenA A501P and A311V had the greatest influence, followed by the N513Y and A501T/V substitutions. Smaller contributions to the cefixime MIC_{pred} were from the PonA L421P, PorB G120, and PenA A517G mutations and a disrupted *mtrR* promoter (meningitidis-like or WHO-P-like), resulting in an adjusted R^2 of 0.783. MIC_{pred} values for the WHO reference strains corresponded to all of the MIC_{pub} and MIC_{pheno} values (Table 1), except for those

TABLE 1 Correlation of antimicrobial MICs of WHO reference strains determined by logistic regression of molecular antimicrobial resistance determinants and phenotypically determined MICs

	MIC (mg/liter) data for drug[b]:																	
	Ciprofloxacin			Tetracycline			Cefixime			Ceftriaxone			Penicillin			Azithromycin		
Strain ID[a]	MIC_{pred}	MIC_{pub}	MIC_{pheno}	MIC_{pred}	MIC_{pub}	MIC_{pheno}	MIC_{pred}	MIC_{pub}	MIC_{pheno}	MIC_{pred}	MIC_{pub}	MIC_{pheno}	MIC_{pred}	MIC_{pub}	MIC_{pheno}	MIC_{pred}	MIC_{pub}	MIC_{pheno}
WHO-F	≤0.004	0.004	0.004	≤0.25	0.25	0.5	≤0.008	<0.016	0.002	≤0.004	0.001	0.001	≤0.125	0.032	0.032	≤0.125	0.125	0.125
WHO-G	0.25	0.125	0.125	32	32	16	0.016	<0.016	0.016	0.008	0.008	0.008	1	0.5	0.5	0.25	0.25	0.25
WHO-K	≥32	>32	64	4	2	2	0.125	0.25	0.25	0.063	0.063	0.063	2	2	2	0.5	0.25	0.5
WHO-L	≥32	>32	32	2	2	2	0.063	0.125	0.063	0.125	0.125	0.125	2	2	2	0.25	0.5	0.25
WHO-M	**0.25**	**2**	**1**	4	2	2	0.031	<0.016	0.016	0.031	0.016	0.016	**128**	≥32	**32**	0.5	0.25	0.5
WHO-N	4	4	4	≥32	16	16	0.016	<0.016	0.008	0.008	0.008	0.008	64	>32	64	0.25	0.25	0.25
WHO-O	**≤0.004**	0.008	**0.016**	4	2	4	0.031	0.016	0.032	0.031	0.032	0.032	128	>32	≥256	0.5	0.25	0.5
WHO-P	≤0.004	0.004	0.004	2	1	1	0.016	<0.016	0.016	0.008	0.008	0.008	0.5	0.25	0.5	4	4	4
WHO-U	≤0.004	0.004	0.004	1	1	2	**0.016**	<0.016	**0.004**	0.008	0.004	0.004	**1**	**0.125**	0.5	4	4	4
WHO-V	16	>32	32	4	4	8	0.031	<0.016	0.032	0.031	0.063	0.063	≥256	>32	≥256	≥256	>256	≥64
WHO-W	≥32	>32	64	4	4	4	0.125	0.25	0.25	0.063	0.125	0.125	**2**	4	**8**	0.5	0.5	0.25
WHO-X	≥32	>32	64	4	2	8	2	4	≥4	1	2	2	**2**	4	**8**	0.5	0.5	0.25
WHO-Y	16	>32	16	4	4	8	≥4	2	≥4	2	2	2	2	1	2	0.5	1	0.5
WHO-Z	≥32	>32	32	2	4	4	2	2	2	1	0.5	0.5	2	2	4	**0.25**	**1**	0.5
Agreement (%)		92.9	85.7		100	100		100	92.9		100	100		92.9	85.7		92.9	100

[a]ID, identifier.

[b]MIC_{pred}, MIC_{pub}, and MIC_{pheno} represent predicted MIC, published MIC by Unemo et al. (18), and phenotypically determined MICs in this study, respectively. Values in bold identify MIC value differences greater than 2 doubling dilutions for each antimicrobial.

TABLE 2 Correlation between MICs determined by logistic regression of molecular antimicrobial resistance determinants and phenotypically determined MICs

Antimicrobial	Data set[a]	No. of isolates matching MIC dilutions of[b]:							% ±1 doubling dilution	Sensitivity (%)[d]	Specificity (%)[d]	% MIC interpretive errors[c]		
		>−2	−2	−1	0	+1	+2	>2				MI	MA	VMA
Ceftriaxone	Canada	0	48	612	363	65	7	0	95.0	NA	100	NB	0	0
	UK/USA	18	61	157	133	51	9	2	79.1	NA	100	NB	0	0
Cefixime	Canada	0	23	204	615	228	22	3	95.6	NA	100	NB	0	0
	UK/USA	10	20	119	126	117	36	3	84.0	NA	100	NB	0	0
Azithromycin	Canada	16	73	187	754	60	2	3	91.4	99.7	81.2	NB	7.7	0.2
	UK/USA	12	14	73	114	134	77	7	74.5	77.3	99.8	NB	0.2	1.2
Ciprofloxacin	Canada	13	70	297	512	181	19	3	90.4	100	100	0.5	0	0
	UK/USA	7	14	31	258	106	5	10	91.7	100	98.8	1.9	0.5	0
Tetracycline	Canada	0	20	386	631	58	0	0	98.2	100	100	13.3	0	0
	UK/USA	8	15	101	152	127	22	6	88.2	99.7	88.0	19.0	0.7	0.2
Penicillin	Canada	13	67	423	487	101	4	0	92.3	100	NA	13.9	0	0
	UK/USA	60	53	125	106	72	15	0	70.3	100	NA	34.1	0.2	0
Overall	Canada	42	301	2,109	3,362	693	54	9	93.8	99.9	97.1	4.6	1.3	0.03
	UK/USA	115	117	606	889	607	164	28	81.3	99.2	99.5	9.2	0.3	0.2

[a]Canadian validation data set, $n = 1,095$, and UK/U.S. international data set from Eyre et al. (28), $n = 431$.
[b]The number of isolates with MIC_{pred} and MIC_{pheno} values that differ by the number of 2-fold dilutions.
[c]The percentage of isolates with minor (MI), major (ME), and very major (VME) interpretative errors for susceptibilities. NB, no CLSI intermediate resistance interpretative breakpoints for these antimicrobials.
[d]NA, too few isolates available with resistant or susceptible interpretative breakpoints were available to provide meaningful specificity or sensitivity values, respectively.

of WHO-F (MIC_{pred}, 0.008 mg/liter; MIC_{pheno}, 0.002 mg/liter) and WHO-U (MIC_{pred}, 0.016 mg/liter; MIC_{pheno}, 0.004 mg/liter). There was 95.6% (1,047/1,095) concordance between the cefixime MIC_{pred} and MIC_{pheno} values to within 1 doubling dilution in the Canadian validation data set and 84.0% (362/431) in the UK/U.S. validation data set. There were no Canadian or UK/U.S. validation isolates with a cefixime MIC_{pheno} of ≥1.0 mg/liter (resistant interpretative breakpoint); therefore, a meaningful sensitivity value could not be calculated. There were no major or very major interpretative errors.

The MIC_{pred} for azithromycin was strongly influenced by the number of 23S rRNA alleles with the A2059G or C2611T mutation, the presence of *mtrR* meningitidis-like and WHO-P-like promoter mutations, and the presence of *ermB* or *ermC*, while lesser contributions were attributed to the *mtrR* −35A deletion, MtrR A39T, and PonA L421P, producing an adjusted R^2 of 0.831. The azithromycin MIC_{pred} values for the panel of 14 WHO reference strains agreed within one doubling dilution to the MIC_{pub} and MIC_{pheno} values, except for strain WHO-Z (MIC_{pred}, 0.25 mg/liter; MIC_{pub}, 1 mg/liter). Agreement within one doubling dilution of the MIC_{pred} and MIC_{pheno} was 91.4% (1,001/1,095) and 74.5% (321/431) for the Canadian and UK/U.S. validation data sets, respectively (Table 2). There was a high degree of sensitivity for resistant predictions (99.7%) but a lower specificity for susceptible predictions (81.2%) due to the relatively large number of susceptible isolates ($n = 69$) that were one MIC_{pheno} dilution below the CLSI resistance breakpoint of 2 mg/liter.

Ciprofloxacin MICs were most influenced by GyrA S91, ParC S87R/I/C, ParC E91, and ParC S86 mutations, with smaller regression coefficients attributed to ParC S87N and ParC S88. The GyrA D95 was found to be an insignificant contributor to increased ciprofloxacin resistance ($P = 0.778$) and was removed from the regression model, resulting in an adjusted R^2 of 0.979. The MIC_{pred} values matched the MIC_{pub} and MIC_{pheno} values for most of the WHO reference strains within one dilution. WHO-M had the GyrA S91F ciprofloxacin resistance determinant, producing a ciprofloxacin MIC_{pred} of 0.25 mg/liter but an MIC_{pub} of 2 mg/liter and MIC_{pheno} of 1 mg/liter; WHO-O had an

MIC_{pred} of 0.004 mg/liter, two dilutions away from the MIC_{pheno} of 0.016 mg/liter. There was 90.4% (990/1,095) agreement of the ciprofloxacin MIC_{pred} and MIC_{pheno} values within one dilution with the Canadian validation data, with sensitivity and specificity both at 100%, 6 minor errors, and no major or very major errors. Agreement with the international UK/U.S. data was 91.7% (395/431), with 100% sensitivity, 98.8% specificity, and 8 minor, 2 major, and no very major interpretative errors.

The best regression model for tetracycline MIC_{pred} had an adjusted R^2 value of 0.812, with *tetM* and the V57M RpsJ amino acid substitution providing the greatest contributions, followed by PorB G120 and A121 mutations, *mtrR* promoter disruptions (*N. meningitidis*-like or WHO-P-like or −35A deletion), and the MtrR A39T substitution. All tetracycline MIC_{pred} values for the WHO reference strain panel were within one dilution of the MIC_{pub} and MIC_{pheno} values. There was a 98.2% (1075/1,095) correlation between the MIC_{pred} and MIC_{pheno} values within one dilution for the Canadian data set, with 100% sensitivity and specificity, 142 minor errors, and no major or very major interpretative errors. There was also a high degree of agreement with the UK/U.S. validation isolates, at 88.2% (380/431), as well as 99.7% sensitivity, 88.0% specificity, 82 minor interpretative errors, 3 major errors, and 1 very major error.

The greatest contributor to penicillin resistance in the regression model was the presence of *bla*, distantly followed by the presence of a disrupted *mtrR* promoter (*N. meningitidis*-like or WHO-P-like), PorB G120, PonA L421P, and PenA N513Y, A517G, and G543S amino acid substitutions for an adjusted R^2 value of 0.720. Among the WHO reference strain penicillin MICs, the MIC_{pred} for WHO-F was 0.125 mg/liter, two dilutions higher than the MIC_{pub} and MIC_{pheno} values (0.032 mg/liter for each); similarly, for WHO-U, the MIC_{pred} was 1 mg/liter, but the MIC_{pub} was 0.125 mg/liter. WHO-W and WHO-X had an MIC_{pred} of 2 mg/liter, within a single dilution of the MIC_{pub} of 4 mg/liter; however, it was two dilutions lower than the MIC_{pheno} of 8 mg/liter. The penicillin MIC_{pred} agreed within one dilution to 92.3% (1,011/1,095) of the Canadian MIC_{pheno}, with 100% sensitivity, 152 minor errors, and no major or very major interpretative errors. The penicillin MIC_{pred} agreement for the UK/U.S. data set was 70.3% (303/431), with 100% sensitivity and specificity and 147 minor errors, 1 major error, and no very major interpretative errors. The relatively large number of minor errors in both validation data sets was due to the very broad CLSI intermediate resistance interpretative breakpoint range for penicillin covering 4 doubling dilutions from 0.125 to 1 mg/liter.

DISCUSSION

Multivariate linear regression modeling successfully estimated the contributions of the commonly recognized molecular antimicrobial resistance determinants in *N. gonorrhoeae* to the MIC values for each antimicrobial investigated. Numerous mutations in *penA* and the presence of recombinant mosaic sequences from other commensal *Neisseria* spp., as well as other contributing factors, including changes to *mtrR*, *porB,* and *ponA*, have been associated with decreased susceptibility of extended-spectrum cephalosporins; however, it was thought that these factors do not fully account for the phenotypes observed and that other factors may be involved (7, 16, 30–40). The optimized regression model indicated that extended-spectrum cephalosporin susceptibility within the data sets of this study can be fully described by the combination of a relatively small number of factors, including five key amino acid changes in PenA, and the presence or absence of the disrupted *mtrR* promoter, a PorB G120 mutation, and/or the PonA L421P mutation.

The PenA A501P had the largest regression coefficient value of approximately 5 for both cefixime and ceftriaxone, corresponding to 5 MIC doubling dilution increments, whereas the A501V and A501T substitutions had coefficients of approximately 1.5 to 2.0, representing 2-fold increases in MICs. Transformation experiments investigating the contributions of various PenA amino acid substitutions to increasing cephalosporin MIC values (41–45) have reported a similar 5-fold MIC increase attributed to the A501P mutation and a 2-fold increase due to the A501T/V mutations. The A311V mutation also was found to contribute significantly to the overall cephalosporin MIC, with regression

coefficients representing 4 doubling dilutions; this is higher than in transformation experiments, where only a 2-fold increase was reported (43). The regression coefficients for the N513Y mutation of 3 and 1.5 for cefixime and ceftriaxone, respectively, corresponded to transformation studies reporting 2-fold increases in cephalosporin MICs (42). It has also been reported that the PenA G453S substitution is more important for ceftriaxone resistance than for cefixime (42), which is reflected in the increased regression coefficient of 0.5 for ceftriaxone compared to an insignificant regression *P* value for cefixime. The regression model predicted ceftriaxone and cefixime MICs matching those expected for the WHO reference strain panel (Table 1) and over 95% of the phenotypically determined MICs among the Canadian validation data set within one doubling dilution.

Azithromycin MIC_{pred} values were most influenced by the A2059G and C2611T 23S rRNA mutations, where each allele with an A2059G mutation contributed 2.6 dilution increments, and each allele with a C2611T mutation contributed 1.3 dilution increments to the predicted MIC. Disruption of the *mtrR* promoter region was also identified as an important contributor to overall azithromycin MIC levels, with the WHO-P-like promoter contributing more to azithromycin resistance than does the *N. meningitidis*-like promoter, with regression coefficients of 4.9 and 3.5, respectively. Although rare, the presence of *ermB* or *ermC* also contributed to MIC values with high regression coefficient values of 2.4 and 3.0, respectively. Smaller contributions to overall azithromycin MIC values were the *mtrR* −35A deletion and the MtrR A39T and PorB G120 amino acid substitutions. The regression results agree with previous reports that describe major macrolide resistance mechanisms in *N. gonorrhoeae*, including 23S rRNA mutations, inhibited regulation of the MtrCDE efflux pump, and smaller contributions from MtrR amino acid substitutions (10, 11, 13, 14). Macrolide resistance has not been previously associated with *porB*; however, the regression analysis generated significant *P* values for the PorB G120 amino acid substitution, and including it in the regression model improved the azithromycin MIC correlations with the WHO reference strains. It is unclear if this may be due to a coincidental statistical association caused by the content of the training data set which is enriched with antimicrobial-resistant isolates, or speculatively, that the altered porin protein may decrease the influx of azithromycin into the cell, as has been described for increased resistance to other hydrophobic antimicrobials and tetracycline (8, 22, 46).

Fluoroquinolone resistance has been strongly associated with GyrA and ParC amino acid substitutions (18–20); however, regression analysis of these reported mutations was able to predict the contribution of each mutation to the overall MIC. While the GyrA D95 mutation seemed to have no influence on ciprofloxacin MICs, they were highly dependent upon the GyrA S91 and the ParC S86, S87, and E91 amino acid substitutions each having regression coefficients greater than 5, and the ParC S88P mutation contributed less, with a coefficient of 1.4. Furthermore, the magnitude of the MIC contribution associated with the ParC S87 mutation was found to be dependent upon the particular amino acid substituted, with S87I, S87IR, and S87C having the greatest influence (coefficient, >4), whereas the S87N mutation contributed less (coefficient, <2).

The presence of *bla* was largest contributor to penicillin MIC values, with a regression coefficient of over 6, and its presence typically resulted in MIC_{pred} values of at least 32 mg/liter. Other more moderate increases to MIC were due to the PonA L421P, PorB G120, and PenA N513Y and A517G mutations, followed by weaker contributions by an *N. meningitidis*-like or WHO-P-like *mtrR* promoter and the PenA G543S substitution. The contributions of molecular determinants to tetracycline resistance generally coincide with those previously described (22, 28), with the presence of *tetM* having the greatest effect, followed by the presence of the RpsJ V57M mutation, a disrupted *mtrR* promoter (*N. meningitidis*-like or WHO-P-like), and smaller contributions from MtrR A39T, PorB G120, and PorB G121.

Predicted MIC values for the six antimicrobials matched 96% of those published for the panel of 14 WHO reference strains (18) within one doubling dilution (Table 1).

Phenotypic MIC values for the WHO reference strains were determined as the modal MIC value from routine quality assurance testing, and the overall MIC_{pred} concordance was 93%. WHO-M possessed the GyrA S91F substitution as the sole fluoroquinolone resistance determinant, which resulted in a ciprofloxacin MIC_{pred} of 0.25 mg/liter. Although this MIC_{pred} was two dilutions lower than the modal MIC_{pheno} of 1 mg/liter during routine testing using WHO-M as a control strain in 10 quality assurance panels, it fell within the range of MICs from 0.5 to 4 mg/liter. Similarly, the PonA L421P and PenA A517G penicillin resistance determinants present in WHO-U produce an MIC_{pred} of 1 mg/liter, two dilutions higher than the phenotypic modal value of 0.25 mg/liter but within the range of MICs of 0.125 to 0.5 mg/liter. WHO-Z possessed only the PorB G120K azithromycin resistance determinant producing a low MIC_{pred} of 0.25 mg/liter, which was two dilutions below the modal phenotypic value of 1 mg/liter (range, 0.5 to 1 mg/liter). These wide ranges of phenotypically derived MIC values reveal the high degree of variability and subjectivity in the nature of phenotypic MIC testing which may explain these discrepancies; however, the presence of additional undescribed factors present in these control strains influencing MIC values cannot be discounted. The regression modeling in this study selected only those factors contributing to increased MIC values by having positive regression coefficients. In the case of the higher-than-expected penicillin MIC_{pred} for WHO-U, it may be possible that there are other factors that interfere with the full expression of resistance factors to decrease MIC values.

There was an overall correlation of 93.8% for MIC_{pred} to MIC_{pheno} within one dilution in the Canadian validation data set, lower than the 81.3% agreement for the UK/U.S. validation data set (Table 2) but higher than the 92.4% correlation of MIC_{pred} comparisons reported by Eyre et al. (28) for the 431 isolates of the UK/U.S. data set (Table S13). In particular, greater MIC_{pred} correlations were attained for cefixime, penicillin, and tetracycline in this study, whereas higher agreement of azithromycin and ciprofloxacin MIC_{pred} was seen in the data set presented by Eyre et al. (28). Correlation rates also varied geographically, with higher agreement with U.S. strains for ceftriaxone and penicillin, whereas for cefixime and azithromycin, agreement was higher with the UK validation data. Lower MIC correlations using the Canadian-based training data set to generate the regression equations with the international data set could be due to different sampling, culturing methods, laboratory testing procedures, interpretation of results, geographical clonal variation, and the inability to confirm phenotypic or sequencing results.

Despite these discrepancies, the comparison of MIC_{pred} to MIC_{pheno} compares favorably to comparison studies of purely phenotypic results. A study in 2015 compared the results of 25 quality assurance proficiency panels for the Canadian National Gonococcal Antimicrobial Susceptibility Comparison Program (47), where the average MIC agreement ranged from 85.6% to 98.9% and interpretation agreed from 85.7% to 98.1% between 9 reference laboratories over a 10-year period. The results from a 2018 comparison of international antimicrobial proficiency panel results from various Caribbean and South American countries (48) reported an overall agreement of $>$90% for MIC results and modal MICs for 5 of the 11 participants, with agreement among the other laboratories ranging from 60.0% to 82.4%. MIC agreement among the participating laboratories for cefixime and azithromycin was $>$90%, whereas for tetracycline, ceftriaxone, and ciprofloxacin, agreement ranged from 84.5% to 89.1%, and for penicillin, overall agreement was 78.8%.

The limitations of the study include that the accuracy and precision of the MIC prediction based on molecular determinants are largely limited by the training data used to generate the regression equations. The training data may include variability due to the subjective nature of phenotypic testing, where the same phenotypes may not always be observed on repeat testing; molecular resistance profile errors and the possible presence of as-yet-unidentified resistance factors affect the formation of the regression model. While using a large training data set to develop the regression model can resolve some discrepancies, some rare resistance patterns, such as very high ceftriaxone and cefixime resistance, are reliant on the availability of a relatively small

number isolates with this phenotype. Furthermore, there may also be some rare resistance determinants that were not present or were present in insufficient numbers to significantly influence the regression model. These limitations can be reduced by increasing the size of the training data with strains from more varied regions of the world and regularly updating the regression model with newly discovered factors and updated coefficient values for currently identified factors. Furthermore, the MIC prediction models described here can be easily generated using the molecular markers discussed in this study with local training phenotypic data sets which may be more applicable to different laboratory testing environments.

The spread of antimicrobial-resistant *N. gonorrhoeae* emphasizes the need for surveillance systems that not only closely track the dissemination of known resistant strains but also promptly detect the novel expansion of resistant clones as they emerge to limit the expansion through sexual networks. As molecular-based genomic techniques become more broadly available not only to identify lineages but also provide additional information regarding molecular antibiotic resistance, virulence, and fitness determinants (27, 49, 50), the MIC-predicting strategy described here can provide a powerful tool to replace traditional phenotypic MIC determination. The ability to acquire detailed antimicrobial resistance information directly from molecular data for use in molecular assays will enhance the monitoring of the dynamics of *N. gonorrhoeae* strains and effectively inform public health interventions to reduce the burden of disease.

MATERIALS AND METHODS

Isolates and antimicrobial susceptibility testing. Multivariate linear regression analysis was performed using Excel 2010 (version 14.0.7151.5001; Microsoft Corp.) on a training data set of 1,264 *N. gonorrhoeae* isolates collected from 1989 to 2018 for which whole-genome sequencing data were available from previous projects (27, 49, 51, 52). Isolates from these projects provided a broad range of ciprofloxacin, azithromycin, cefixime, ceftriaxone, penicillin, and tetracycline MICs and consisted of 510 Canadian isolates enriched with isolates having decreased cephalosporin susceptibility (49), a set of 429 Canadian and 47 Dutch isolates enriched for azithromycin resistance (27, 51), 117 Canadian isolates with diverse antimicrobial resistance used for the development of a real-time PCR antimicrobial resistance assay (52), and a convenience sample of 161 other diverse Canadian isolates selected from a national enhanced gonococcal antimicrobial resistance surveillance project (https://www.canada.ca/en/public-health/services/publications/drugs-health-products/national-surveillance-antimicrobial-susceptibilities-neisseria-gonorrhoeae-annual-summary-2017.html). Fourteen WHO reference strains (18) were included to enrich the training data with high-level cephalosporin phenotypic MICs, and 2 strains from Eyre et al. (28) (SRA accession numbers ERR191763 and ERR191769) with the relatively rare ParC S87I substitution were also added to enhance ciprofloxacin MIC regression training. The validation data included a data set of 1,095 Canadian isolates collected from 2013 to 2019 and an international validation data set described by Eyre et al. (28), with 431 *N. gonorrhoeae* isolates with complete antimicrobial resistance data from the United Kingdom ($n = 245$) and United States ($n = 186$) obtained from the Sequence Read Archive of the NCBI.

Antimicrobial susceptibilities to ciprofloxacin, azithromycin, cefixime, ceftriaxone, penicillin, and tetracycline for the Canadian and U.S. data sets were determined using the agar dilution method, according to Clinical and Laboratory Standards Institute (CLSI) guidelines (53), and those for the UK were determined by the GRASP method (28). MIC resistance interpretations were based on the CLSI criteria for penicillin (MIC, ≥2.0 mg/liter), tetracycline (MIC, ≥2.0 mg/liter), and ciprofloxacin (MIC, ≥1.0 mg/liter) (53). WHO guidelines were used to define cefixime and ceftriaxone resistance at MICs of ≥0.25 mg/liter and ≥0.5 mg/liter, respectively (54), and the resistance breakpoint for azithromycin was an MIC of ≥2.0 mg/liter (55).

Molecular analysis. Molecular antimicrobial resistance determinants were identified *in silico* from whole-genome sequencing data, as previously described (27, 28, 49). The *mtrR* promoter disruptions (−35A deletion, mosaic *N. meningitidis*-like and WHO-P disrupted sequences), presence of the *ermB* and *ermC* genes, MtrR A39T and G45D mutations, and 23S rRNA A2059G and C2611T mutations (23S rRNA mutations are *Escherichia coli* numbering corresponding to A2045G and C2597T in *N. gonorrhoeae* NCCP11945, respectively) were included as azithromycin susceptibility determinants. Tetracycline resistance markers included the presence of *tetM*, *mtrR* promoter disruptions, and the RpsJ V57M, MtrR A39T and G45D, PorB G120 and A121, and PonA L421P substitutions. GyrA amino acid substitutions S91 and D95 and ParC D86, S87, S88, and E91 substitutions were analyzed as ciprofloxacin resistance determinants. Penicillin and cephalosporin resistance factors analyzed included the presence of *bla*, *penA* mutations, *mtrR* promoter disruptions, and MtrR A39T and G45D, PorB G120 and A121, and PonA L421P substitutions.

Multivariate regression analysis. Multivariate regression analyses (56) were performed using Excel 2010 (version 14.0.7151.5001; Microsoft Corp.) to determine the relationship of the molecular antimicrobial resistance determinants contained in an isolate to the phenotypically determined MIC values for

azithromycin, penicillin, ceftriaxone, cefixime, ciprofloxacin, and tetracycline (27, 28). The doubling phenotypic MIC values were standardized to exact doubling dilutions (512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, 0.0078125, 0.00390625, 0.001953125, and 0.000976563 mg/liter), converted to a linear increment scale using a formula, phenotypic MIC increment = $\log_2$(standardized MIC), and used as the dependent variable in the regression analysis. Molecular markers were used as independent variables and represented by a presence or absence with a value of 1 or 0, respectively, except the variable for the 23S rRNA A2059G and C2611T mutations, which corresponded to the number of alleles with a respective mutation. A regression model for each antimicrobial was built from a preliminary analysis that included all independent variables, followed by stepwise removal of variables with relatively high individual *P* values and those causing little change in the adjusted coefficient of determination (R^2) value (see the supplemental material for metadata and MS Excel regression outputs). An adjusted R^2 value (95% confidence interval) of 0.0 to 0.1 was considered no correlation to a very weak correlation, 0.2 to 0.4 was a weak correlation, 0.5 to 0.7 was a moderate correlation, 0.8 to 0.9 was a strong correlation, and >0.9 was a very strong correlation. Predicted MIC (MIC_{pred}) values for each antimicrobial were calculated by first calculating the predicted MIC increment by summing the regression intercept and independent variable coefficients for each isolate, rounding fractional values up or down to the nearest whole integer, and then converting this value back to a doubling MIC value using the following formula: predicted MIC value = $2^{\text{predicted MIC increment}}$. Individual *P* values of <0.05 for the independent variables at a confidence interval of 95% were considered significant.

Sensitivity and specificity for the MIC_{pred} were based on agreement of the antimicrobial susceptibilities as predicted by the molecular markers to that confirmed by traditional phenotypic testing, with true positive (TP) defined as resistant predicted and phenotypic MICs, false negative (FN) defined as resistant predicted MICs and susceptible phenotypic MICs, true negative (TN) defined as susceptible predicted and phenotypic MICs, and false positive (FP) defined as a susceptible predicted MICs and resistant phenotypic MICs. Calculations were performed as follows: sensitivity (SENS) = TP/(FN + TP) × 100, and specificity (SPEC) = TN/(FP + TN) × 100 (57). Antimicrobial resistance interpretative errors were defined as follows: a minor error (MI) was when the MIC_{pred} corresponded to intermediate resistance and phenotypically derived MICs (MIC_{pheno}) corresponded to either susceptible or resistance interpretations and vice versa, a major error (ME) was when the MIC_{pred} corresponded to a resistant interpretation and the MIC_{pheno} was susceptible, and a very major error (VME) was when the MIC_{pred} was susceptible and the MIC_{pheno} was resistant.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.4 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.2 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.1 MB.

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