

Antimicrobial susceptibility of Canadian isolates of *Helicobacter pylori* in Northeastern Ontario

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NF Eng, G Ybazeta, K Chapman, et al. Antimicrobial susceptibility of Canadian isolates of *Helicobacter pylori* in Northeastern Ontario. *Can J Infect Dis Med Microbiol* 2015;26(3):137-144.

BACKGROUND: *Helicobacter pylori* plays a significant role in gastritis and ulcers. It is a carcinogen as defined by the WHO, and infection can result in adenocarcinomas and mucosa-associated lymphoid tissue lymphomas. In Canada, rates of antimicrobial resistance are relatively unknown, with very few studies conducted in the past 15 years.

OBJECTIVE: To examine rates of resistance in Sudbury, Ontario, compare antimicrobial susceptibility methods and attempt to determine the molecular basis of antibiotic resistance.

METHODS: Patients attending scheduled visits at Health Sciences North (Sudbury, Ontario) provided gastric biopsy samples on a volunteer basis. In total, 20 *H pylori* isolates were collected, and antimicrobial susceptibility testing (on amoxicillin, tetracycline, metronidazole, ciprofloxacin, levofloxacin and clarithromycin) was conducted using disk diffusion and E-test methods. Subsequently, genomic DNA from these isolates was sequenced to detect mutations associated with antimicrobial resistance.

RESULTS: Sixty-five percent of the isolates were found to be resistant to at least one of the listed antibiotics according to E-test. Three isolates were found to be resistant to ≥ 3 of the above-mentioned antibiotics. Notably, 25% of the isolates were found to be resistant to both metronidazole and clarithromycin, two antibiotics that are normally prescribed as part of first-line regimens in the treatment of *H pylori* infections in Canada and most of the world. Among the resistant strains, the sequences of 23S ribosomal RNA and *gyrA*, which are linked to clarithromycin and ciprofloxacin/levofloxacin resistance, respectively, revealed the presence of known point mutations associated with antimicrobial resistance.

CONCLUSIONS: In general, resistance to metronidazole, ciprofloxacin/levofloxacin and clarithromycin has increased since the studies in the early 2000s. These results suggest that surveillance programs of *H pylori* antibiotic resistance may need to be revisited or improved to prevent antimicrobial therapy failure.

Key Words: Antimicrobial susceptibility; *Helicobacter pylori*; Northern Ontario; Resistance

Helicobacter pylori is a bacterial human pathogen that preferentially colonizes the stomach and is found in 50% of the world's population, causing dyspepsia, gastritis and peptic ulcers (1). The WHO has classified this organism as a class I carcinogen that can cause stomach cancers, which, after diagnosis, has only a 27% survival rate (2-4). In Canada, the prevalence of *H pylori* is approximately 20% to 30% (5,6). However, in developing countries, rates

La susceptibilité antimicrobienne d'isolats canadiens d'*Helicobacter pylori* au nord-est de l'Ontario

HISTORIQUE : L'*Helicobacter pylori* contribue énormément à la gastrite et aux ulcères. L'OMS le définit comme un cancérigène, et l'infection peut provoquer l'apparition d'adénocarcinomes et de lymphomes de tissus lymphoïdes associés aux muqueuses. Au Canada, on connaît relativement peu les taux de résistance antimicrobienne, car très peu d'études ont été réalisées sur le sujet depuis 15 ans.

OBJECTIF : Examiner les taux de résistance à Sudbury, en Ontario, comparer les méthodes de susceptibilité antimicrobienne et tenter de déterminer le fondement biologique de la résistance antibiotique.

MÉTHODOLOGIE : Les patients qui allaient à un rendez-vous prévu au Health Sciences North de Sudbury ont remis les résultats de biopsies gastriques sur une base volontaire. Au total, 20 isolats de *H pylori* ont été recueillis, et les tests de susceptibilité antimicrobienne (à l'amoxicilline, à la tétracycline, au métronidazole, à la ciprofloxacine, à la lévofloxacine et à la clarithromycine) ont été effectués au moyen de la diffusion sur disque et de l'essai E. L'ADN génomique de ces isolats a ensuite été séquençé pour déceler les mutations associées à la résistance antimicrobienne.

RÉSULTATS : Selon l'essai E, 65 % des isolats étaient résistants à au moins un des antibiotiques énumérés. Notamment, 25 % des isolats étaient résistants à la fois au métronidazole et à la clarithromycine, tous deux normalement prescrits en première ligne pour traiter les infections à *H pylori* au Canada et dans la plupart des régions du monde. Parmi les souches résistantes, les séquences d'ARN ribosomique 23S et de *gyrA*, qui sont liées à la résistance à la clarithromycine et à la ciprofloxacine-lévofloxacine, respectivement, révélaient la présence de mutations de points connus associés à une résistance antimicrobienne.

CONCLUSIONS : En général, la résistance au métronidazole, à la ciprofloxacine-lévofloxacine et à la clarithromycine a augmenté depuis les études réalisées au début des années 2000. D'après ces résultats, il faudra peut-être revoir ou améliorer les programmes de surveillance de l'antibiorésistance au *H pylori* pour prévenir l'échec du traitement antimicrobien.

can be as high as 80% (7). As of 2007, the incidence in the province of Ontario was approximately 23%, with men twice as likely to be infected as women (29.4% versus 14.9%) (5). Infection is strongly correlated with crowding, smoking, diet and poor sanitation (8-11). Unfortunately, such conditions exist within the most vulnerable populations in Canada; specifically, some First Nations communities have demonstrated rates as high as 95% (12).

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Eradication of *H pylori* is dependent on successful treatment with a proton pump inhibitor (PPI), such as omeprazole, lansoprazole and rabeprazole, and at least two antibiotics among clarithromycin (CLR), metronidazole (MTZ), amoxicillin (AMX) and tetracycline (TET) (13-16). In Canada, one of the standard recommended first-line regimens is a triple therapy consisting of a PPI, CLR, and either AMX or MTZ for up to 14 days (17). Alternatively, a quadruple regimen including a PPI, bismuth, MTZ and TET for up to 14 days may be required (18). Initially, both regimens had eradication rates >90% in 2000 (19), but this had dropped to 70% 10 years later (20), well below the recommended 80% outlined by Maastricht IV guidelines (21). A major contributor to the decline in efficacy is the increasing prevalence of antimicrobial resistance through selection, mutation and the development of emerging efflux pumps. This compels the necessity of susceptibility testing to manage the infection effectively (13). In one study, nearly 50% of patients in Germany were unable to eradicate *H pylori* because of resistance to both CLR and MTZ (22). In another study, nitroimidazole resistance was a contributing factor of eradication rates dropping by 15% to 50% (23). A recent study evaluated whether a new regimen of AMX plus a high dose of lansoprazole can achieve ≥90% eradication of *H pylori* (24). It was believed that the high PPI dose could affect the stomach environment and increase antimicrobial susceptibility; however, the desired eradication rate was not achieved. With mounting evidence that antimicrobial resistance affects clinical outcomes, there is a need to place more emphasis on a surveillance program to monitor resistance to guide the treatment of *H pylori* infections.

One of the mechanisms by which *H pylori* acquires resistance to antibiotics is through vertical transmission of mutations. For example, it is well documented that CLR resistance is linked to point mutations at nucleotide A2142 and A2143 in the 23S ribosomal (r)RNA gene (*rm*) (25,26). Resistance to TETs were observed when all three nucleotides at positions 926 to 928 of the 16S rRNA gene were mutated (27,28). However, determining whether newly discovered mutations are responsible for antimicrobial resistance is a challenging task. Care must be taken because several mutations can appear in any given gene, and only through methodical evaluation (eg, site-directed mutagenesis of a susceptible wild-type *H pylori* strain) can a proper conclusion be made about a particular mutation leading to resistance.

Recent reporting of *H pylori* in Canada has been sporadic, with the most recent comprehensive epidemiological analyses on prevalence and antimicrobial resistance in Canada as a whole being published in 2000 (29) and 2004 (30). More recently, a study in the Arctic region of Canada has examined rates of antimicrobial resistance in an Aboriginal community in Aklavik, Northwest Territories (31). Additionally, there was a report in 2007 about the prevalence of *H pylori* in Ontario (5), and in a First Nations community in Sioux Lookout in Northwestern Ontario (32). In 1998, studies in Alberta showed that MTZ resistance was 14%, while CLR resistance was 3% (33). While it is currently unknown how *H pylori* has progressed in prevalence or whether antimicrobial resistance has increased across Canada, *H pylori* resistance to CLR across Europe doubled from 9% to 18% between 1998 and 2008, suggesting that antimicrobial resistance has increased globally (21).

The present pilot study provides a small glimpse into *H pylori* antimicrobial susceptibility in Northeastern Ontario by examining rates of antibiotic resistance for patients who attended Health Sciences North (HSN) in Sudbury, Ontario, for care. Different methods to determine antimicrobial resistance are compared and molecular detection of mutations known to be associated with antibiotic resistance are examined. Our introductory findings show increased local rates of antimicrobial resistance. As such, the extent of rising antimicrobial resistance rates of *H pylori* should be examined nationally and internationally as a means to prevent further treatment failure.

METHODS

Gastric biopsy collection, processing and culturing of *H pylori*

The HSN Research Ethics Board in Sudbury, Ontario, approved the research protocol. Patients who were scheduled for gastroscopies as

part of their regular health care were asked for informed consent to collect blood and additional biopsy samples for the present study. In total, 301 patients were recruited to the study (117 men and 184 women); 127 participants were ≥60 years of age. Demographically, 280 identified themselves as Caucasian, 18 as First Nations, two as of African descent and one as of Indian descent. At HSN, gastric biopsies are typically sent for histology to identify *Helicobacter*-like organisms using Giemsa staining. Presence of these organisms would then prompt physicians to prescribe standard levels of treatment with a follow-up later. Physicians in the endoscopy unit who participated in the present study extracted additional biopsies and placed them into tubes containing transport medium consisting of thioglycollate medium (Oxoid, Canada), as per the manufacturer's instructions. To increase the likelihood of recovering *H pylori*, the biopsies were processed within 20 min of biopsy collection (E Altman, National Research Council of Canada, personal communication).

Briefly, biopsies were ground between two glass slides to break up the tissue and then plated on Columbia blood agar (BD Difco, USA) supplemented with a final concentration of 7% defibrinated horse blood (Quelab, Canada) and antibiotics (Sigma-Aldrich, Canada) as previously described (34). The plates were then placed into the Invivo₂ 500 Hypoxia Workstation (Ruskin Technology Ltd, United Kingdom) at 37°C under microaerophilic conditions (5% O₂, 10% CO₂, 85% N₂) for a minimum of 48 h to two weeks to facilitate the culture of *H pylori*. Colonies were streaked for isolation on fresh media and subsequently cultured under the same conditions for 48 h to 72 h. When sufficient growth had occurred, samples were prepared for storage in Brain Heart Infusion broth (BD Difco) supplemented with 20% glycerol (Fisher Scientific, Canada) at -80°C. Enough bacteria were resuspended to cause turbidity in the freezing media. *H pylori* isolates were minimally cultured (two to five times due to varying rates of growth) to prevent the introduction of mutations under selective pressure.

Biochemical and polymerase chain reaction identification of *H pylori*

A KOH test was performed to rapidly determine whether isolated bacteria were Gram negative (35). A small amount of bacteria on a loop was added to a drop of 3% KOH (Sigma-Aldrich, Canada) on a microscope slide. A positive reaction (ie, Gram negative) was indicated if the mixture became viscous within 30 s and a string of the mixture was observed by raising the loop approximately 1 cm from the slide.

H pylori colonies were identified by positive catalase, urease and oxidase tests as previously described (36). Catalase was determined to be present if a drop of 3% H₂O₂ (Sigma-Aldrich) on a slide with bacteria led to effervescence. A urease test was considered to be positive if bacteria introduced in a solution of Brucella broth (BD Difco), urea (Sigma-Aldrich) and phenol red (Sigma-Aldrich) at pH 6.8 to 7.0 (yellow colour) turned pink or red within 24 h. Finally, a positive reaction for oxidase was indicated when bacteria smeared on filter paper dampened with N,N,N,N-tetramethyl-p-phenylenediamine dihydrochloride (Kovacs reagent, Sigma) left a purple colour on the filter paper.

The identity of *H pylori* was confirmed and further characterized by amplifying 16S rRNA, *cagA*, *ureC* and *ureA*, with primers listed in Table 1 using the Multiplex PCR Kit (Qiagen, Canada) as per the manufacturer's instructions in the VapoProtect gradient thermocycler (Eppendorf, Canada). Expected size amplicon lengths of 522, 294, 400 and 411 base pairs, respectively, were resolved on a 2% agarose gel using the PowerPac HC (BioRad, Canada).

Antimicrobial susceptibility

Antimicrobial susceptibility testing was conducted using two methods (disk diffusion and E-test). Colonies from two- to three-day-old pure culture of *H pylori*, grown on nonselective Mueller-Hinton (MH) agar (BD Difco) plates and supplemented with 5% defibrinated horse blood (Quelab), were resuspended in phosphate-buffered saline to a McFarland standard of 2 (approximately 6×10⁸ cfu/mL) for the disk

diffusion testing, and a standard of 3 (approximately 1.0×10^9 cfu/mL) for the E-test. *H pylori* 26695 was used as a negative control because the strain is susceptible to all of the antibiotics tested in the present study (37,38).

The disk diffusion test followed the guidelines as set out by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), April 2013, for microaerophilic bacteria (39). Bacterial suspensions were applied to the MH blood agar plates using a sterile cotton swab within 30 min, and the antibiotic disks were applied within 15 min on three separate occasions and averaged. The plates were incubated in the microaerophilic chamber at 37°C for three days, at which time the zones of inhibition were measured in millimetres. The disks (Oxoid) used were AMX (10 µg/disk), CLR (15 µg/disk), TET (30 µg/disk), MTZ (5 µg/disk), ciprofloxacin (CIP; 5 µg/disk), and levofloxacin (LVX; 5 µg/disk) as suggested by EUCAST and the Clinical and Laboratory Standards Institute standards for antibiotic content. Resistance was determined when zones of inhibition were ≤ 25 mm for AMX, ≤ 30 mm for CLR and TET, ≤ 16 mm for MTZ (39), and ≤ 30 mm for CIP (40) and LVX (41).

For the E-test, the same inoculation procedure was applied except with the use of a 3 McFarland standard. Each E-test strip (BioMérieux, Canada) of the same antibiotics as above was applied onto the MH blood agar plates, incubated similarly as with the disk diffusion test, and readings were taken after three days, according to the manufacturer's instructions. The breakpoints used to classify strains as susceptible or resistant are as follows, according to the latest EUCAST recommendations as of January 2014 (42): ≤ 0.12 µg/mL susceptible, > 0.12 µg/mL resistant for AMX; ≤ 1 µg/mL susceptible, > 1 µg/mL resistant for CIP, LVX and TET; ≤ 0.25 µg/mL susceptible, > 0.5 µg/mL resistant for CLR; and ≤ 8 µg/mL susceptible, > 8 µg/mL resistant for MTZ. The E-test evaluation was conducted in duplicate on two different occasions.

Genomic DNA extraction

H pylori was grown in Columbia broth (Quelab) supplemented with 10% fetal bovine serum with shaking at 100 rpm using a MaxQ 6000 incubated shaker (Thermo Scientific, USA) under microaerophilic conditions at 37°C for 48 h to 72 h. Subsequently, the broth culture was centrifuged at $1700 \times g$ for 20 min. The supernatant was removed, and the pellet was resuspended in phosphate-buffered saline and then centrifuged again. Following removal of the supernatant, resuspension buffer (50 mM Tris-HCl, pH 7.5 [BioShop, Canada], 10 mM EDTA [BioShop], 100 mg/mL RNase A [Fermentas, Canada]) was added with an equal volume of a mixture of a 25:24:1 ratio of phenol (Fisher), chloroform (BioShop) and isoamyl alcohol (BioShop), respectively (PCI). The mixture was shaken for 2 min to 3 min and then centrifuged at $15000 \times g$ in the Sorvall Legend Micro 17 centrifuge (Fisher) for 10 min. Twice, the supernatant was carefully retrieved and then another equal volume of PCI was added, mixed and centrifuged as previously described. RNase A/T1 (Fermentas) was then added to the supernatant and allowed to incubate in a 37°C water bath for 1 h. PCI was once more added to remove the RNase. Approximately 2.5 to 3 times the volume of an ethanol mix (0.16 M of sodium acetate in 500 mL absolute ethanol) was added to initiate DNA precipitation. After incubation at -20°C overnight, the solution was centrifuged again at $15000 \times g$ to pellet the precipitated DNA. The pellet was then washed with 70% ethanol and dried using a Hetovac VR-1 vacuum centrifuge (Heto Lab Equipment, Denmark). DNase- and RNase-free water was used to solubilize the DNA. The purity and concentration of the DNA was verified using agarose gel electrophoresis and a Nanodrop 2000c Spectrophotometer (Fisher), respectively.

Sequencing of H pylori genome

All genome sequences generated at the Advanced Medical Research Institute of Canada and HSN were produced using an Ion Torrent PGM (Life Technologies, Canada) sequencing technology.

TABLE 1
Primers used for polymerase chain reaction

Gene	Primer sequence (5' → 3')	Reference
16S rRNA	GCTAAGAGATCAGCCTATGTCC	71
	TGGCAATCAGCGTCAGGTAATG	
<i>cagA</i>	AATACACCAACGCCTCCAAG	72
	TTGTTGCCGCTTTTGCTCTC	
<i>ureC</i>	AAGCTTTTAGGGGTGTTAGGGGTTT	73
	AAGCTTACTTTCTAACACTAACGC	
<i>ureA</i>	GCCAATGGTAAATTAGTT	74
	CTCCTTAATTGTTTTAC	

rRNA Ribosomal RNA

The following gene sequences, obtained from GenBank, are involved with antimicrobial resistance and were used to query the entire genomes (43): 16S rRNA (TET) GI: 6626253, 23S rRNA (CLR) GI: 881379, gyrase A (*gyrA*, CIP/LVX) GI: 12057207, penicillin-binding protein 1A (*pbp1A*, AMX) GI: 6626253 and oxygen-insensitive NADPH nitroreductase (*rdxA*, MTZ) GI: 308035684. BLAST v2.2.29+ (44) searches were performed with each gene sequence against each *H pylori* genome. Genome contigs were manually inspected to check for artifacts in the extracted gene sequences using Gap5 v1.2.14-r (45). Extracted genes were saved in FASTA format and visualized in Seaview v4.5.0 (46). Gene alignments were performed using MUSCLE v3.8.31 (47). Each alignment was inspected for stop codons, insertions/deletions and frame shifts. The final alignments for the coding sequences were performed following their codon structure.

RESULTS

Patient recruitment, collection and identification of H pylori

Between October 2012 and November 2013, 301 patients volunteered to participate in the present study to provide gastric biopsy samples. Twenty of these participants (13 male, seven female) tested positive for *H pylori*. Biochemical testing on the isolated bacteria indicated positive reactions for urease, catalase and oxidase. In addition, polymerase chain reaction (PCR) was conducted on the bacterial isolates and 19 of 20 were found to have fragments of *ureA*, *ureC* and 16S rRNA. While the latter two amplicons were detected from the isolate from participant 60, *ureA* was only detected upon sequencing. Only seven of 20 indicated the presence of *cagA* according to PCR. According to the pathology reports, nearly all the patients had gastritis, while four of these patients also had metaplasia or hyperplasia (Table 2). One pathology report was not available. At the time of biopsy, 90% (18 of 20) of the positively identified patients were ≥ 50 years of age.

Disk diffusion assay

The antimicrobial resistance of the 20 *H pylori* isolates to AMX, TET, MTZ, CIP, LVX and CLR is listed in Table 3 as the average zone of inhibition, measured in millimetres. Results are also listed in Table 4 as being susceptible or resistant. Of 20 isolates, eight were resistant for MTZ (40%), six were resistant for CIP and LVX (30%), and eight were resistant for CLR (40%). None of the isolates were resistant to AMX or TET. Multiple resistances, outside of CIP and LVX (fluoroquinolones), were also observed. Two of 20 isolates (10%) were resistant to both MTZ and to the fluoroquinolones, three (15%) were resistant to CLR and the fluoroquinolones, five (25%) were resistant to MTZ and CLR, while two (10%) were resistant to MTZ, CLR and to the fluoroquinolones.

Minimum inhibitory concentration determination using E-test

The disk diffusion method alone is not sufficient to evaluate *H pylori* antimicrobial resistance because it is not yet standardized. As such, the E-test, following the manufacturer's instructions, was performed in addition to determine minimum inhibitory concentration (MIC) as recommended by EUCAST. Table 4 shows the resulting MIC determination

TABLE 2
Resistance profile of 20 *Helicobacter pylori* isolates according to disk diffusion and E-test methods

Isolate number	Pathology	AMX		TET		MTZ		CIP		LVX		CLR	
		10 µg*	MIC†	30 µg*	MIC†	5 µg*	MIC†	5 µg*	MIC†	5 µg*	MIC†	15 µg*	MIC†
45	No pathology performed	S	<0.016	S	<0.023	S	<0.75	R	>32	R	<8	R	>256
60	Chronic gastritis, metaplasia	S	<0.023	S	<0.094	R	>256	S	<0.064	S	<0.094	S	<0.19
63	Chronic gastritis	S	<0.016	S	<0.032	R	<0.75	S	<0.125	S	<0.125	S	<0.064
66	Chronic gastritis, hyperplasia	S	<0.016	S	<0.25	R	>256	R	>32	R	>32	R	>256
103	Chronic gastritis	S	<0.023	S	<0.019	S	<0.75	S	<0.38	S	<0.25	S	<0.047
104	Gastritis	S	<0.032	S	<0.032	R	<64	S	<0.19	S	<0.19	R	>256
163	Chronic gastritis	S	<0.023	S	<0.125	R	>256	S	<0.75	S	<0.5	R	>256
170	Chronic gastritis	S	<0.016	S	<0.047	S	<0.5	S	<0.019	S	<0.019	R	>256
173	Acute and chronic gastritis	S	<0.016	S	<0.047	S	<0.38	S	<0.25	S	<0.25	S	<0.064
181	Chronic gastritis	S	<0.016	S	<0.125	S	<0.5	S	<0.125	S	<0.125	S	<0.047
195	Chronic gastritis	S	<0.016	S	<0.032	R	>256	S	<0.125	S	<0.094	R	>256
207	Chronic gastritis	S	<0.032	S	<0.023	S	<0.094	S	<0.064	S	<0.125	S	<0.094
209	Chronic gastritis	S	<0.016	S	<0.032	S	<0.125	R	>32	R	>32	S	<0.047
227	Chronic gastritis	S	<0.016	S	<0.094	S	<0.047	R	>32	R	<6	S	<0.023
236	Chronic gastritis	S	<0.016	S	<0.023	S	<0.38	S	<0.19	S	<0.25	S	<0.032
239	Chronic gastritis	S	<0.064	S	<0.125	R	<48	R	>32	R	>32	R	<96
243	Chronic gastritis, metaplasia	S	<0.032	S	<0.25	R	>256	S	<0.38	S	<0.38	S	<0.094
263	Chronic gastritis, metaplasia	S	<0.064	S	<0.047	S	<0.75	S	<0.25	S	<0.19	R	>256
283	Chronic gastritis	S	<0.032	S	<0.25	S	<1	R	>32	R	>32	S	<0.023
288	Chronic gastritis	S	<0.016	S	<0.023	S	<0.5	S	<0.094	S	<0.125	S	<0.023

*Per disk; †µg/mL. AMX Amoxicillin; CIP Ciprofloxacin; CLR Clarithromycin; LVX Levofloxacin; MIC Minimum inhibitory concentration; MTZ Metronidazole; R Resistant; S Susceptible; TET Tetracycline

TABLE 3
Average zone diameters (in mm) of inhibition according to disk diffusion

Isolate number	AMX	TET	MTZ	CIP	LVX	CLR
45	58.0	54.0	20.7	6.0	14.7	6.0
60	53.3	55.7	9.3	46.0	44.7	54.3
63	62.0	60.3	14.0	47.7	46.7	50.7
66	59.3	50.0	6.0	6.0	6.0	7.7
103	54.7	52.0	19.0	39.7	38.7	52.7
104	53.7	56.7	7.0	43.0	39.7	7.3
163	44.3	58.0	6.0	36.3	34.7	6.0
170	56.7	57.7	16.5	39.3	38.0	6.0
173	56.0	51.7	30.0	39.3	37.7	46.3
181	52.0	40.0	26.3	40.3	44.0	55.0
195	54.7	61.3	6.0	47.3	46.0	6.0
207	60.3	66.7	47.7	49.3	49.0	60.7
209	59.7	57.0	26.7	6.0	6.0	48.3
227	56.7	50.3	16.7	6.0	18.0	48.3
236	56.7	61.7	35.7	44.7	41.0	52.7
239	49.7	52.0	6.0	6.0	15.0	6.0
243	65.0	53.7	6.0	39.0	40.7	54.0
263	51.3	64.0	32.3	44.7	44.0	6.0
283	54.7	52.7	22.3	6.0	6.0	59.7
288	60.3	63.3	23.3	45.3	45.3	57.3

AMX Amoxicillin; CIP Ciprofloxacin; CLR Clarithromycin; LVX Levofloxacin; MTZ Metronidazole; TET Tetracycline

using E-test strips. In evaluating AMX, the MIC was 0.064 µg/mL in two isolates, 0.032 µg/mL in four isolates, 0.023 µg/mL in three isolates and 0.016 µg/mL in 11 isolates. Because the breakpoint for AMX is 0.12 µg/mL, all of the isolates were deemed to be susceptible to AMX. All of the isolates were also susceptible to TET; the breakpoint is 1 µg/mL and the MIC values for all of the isolates were ≤0.25 µg/mL. Of the 20 isolates, seven were found to be resistant to MTZ (35%) because their MIC values were ≥48 µg/mL, which is higher than the breakpoint value of 8 µg/mL.

Quinolone resistance was observed in six isolates (30%), which had MIC values much greater (>6 µg/mL) than the 1 µg/mL breakpoint. Finally, CLR testing showed that seven isolates had the maximum MIC values that were listed on the E-test strip (256 µg/mL), while one was 96 µg/mL, giving rise to a total of eight resistant isolates (40%) that were well above the 0.5 µg/mL breakpoint. There was excellent correspondence between disk diffusion and E-test; however, the isolate from participant 63 was not resistant to MTZ according to E-test, while disk diffusion results suggested resistance. Patterns of multiple resistances were nearly identical to those found by disk diffusion except for those related to isolate 63. There was at least one antibiotic resistance found in 13 of the isolates (65%).

Detection of mutations in *H pylori* genes associated with antimicrobial resistance

To corroborate these data on antimicrobial susceptibility, five *H pylori* genes, which have been documented to be associated with antimicrobial resistance when mutated, were examined. These genes (AMX – *pbp1a*; TET – 16S rRNA; MTZ – *rdxA*; CIP/LVX – *gyrA*; CLR – 23S rRNA) were compared with the sequences from the isolates in the present study (Table 5). The genomes collected were originally for another project at the authors' laboratory; therefore, only the five genes were extracted for analysis.

Seven of the eight strains that were found to be resistant to CLR showed a point mutation, A2143G, in the 23S rRNA sequence, while the other strain from participant 263 revealed an A2142G mutation (Table 5). These mutations were not detected among the strains susceptible to CLR. Six strains that were resistant to CIP and LVX were also found to have mutations. Five had the G271A point mutation in the *gyrA* gene, while one isolate had an A272G mutation. The susceptible strains did not possess these mutations. Interestingly, while many strains were found to be resistant to MTZ, only two showed unique mutations to the *rdxA* gene, which has not been previously documented. One showed a C16T point mutation that led to a stop codon, and an insert at nucleotide 121 with the sequence GAAATCGCC, which kept the remainder of the sequence in frame. No other mutations that have been documented to be involved with resistance were detected in the other resistant strains. There were amino acid mutations detected in the *pbp1a* gene that

TABLE 4
Comparison of antimicrobial susceptibility according to disk diffusion and E-test

Antimicrobial agent	Disk diffusion			E-test	
	Isolates, n (%)		Range, µg/mL	Isolates, n (%)	
	Susceptible	Resistant		Susceptible	Resistant
Amoxicillin	20 (100)	0 (0)	0.016–256	20 (100)	0 (0)
Tetracycline	20 (100)	0 (0)	0.016–256	20 (100)	0 (0)
Metronidazole	12 (60)	8 (40)	0.016–256	13 (65)	7 (35)
Ciprofloxacin	14 (70)	6 (30)	0.002–32	14 (70)	6 (30)
Levofloxacin	14 (70)	6 (30)	0.002–32	14 (70)	6 (30)
Clarithromycin	12 (60)	8 (40)	0.016–256	12 (60)	8 (40)

TABLE 5
Molecular detection of *Helicobacter pylori* mutations that may be linked to antimicrobial resistance

Isolate number	23S rRNA		gyrA		rdxA		pbp1A	
	CLR S/R	Mutation	CIP/LVX S/R	Mutation	MTZ S/R	Mutation	AMX S/R	Mutation
45	R	A2143G (26)	R	G271A (57)	S		S	V45I (64), V16I (64), S543R (66)
60	S		S		R	nd	S	V16I, S543R
63	S		S		R	nd	S	
66	R	A2143G	R	G271A	R	nd	S	
103	S		S		S		S	S543T (present study)
104	R	A2143G	S		R	nd	S	V16I, S543R
163	R	A2143G	S		R	nd	S	S543N (present study)
170	R	A2143G	S		S		S	S543R
173	S		S		S		S	
181	S		S		S		S	S543R
195	R	A2143G	S		R	C16T (stop) (present study)	S	V16I, S543R
207	S		S		S		S	S543N
209	S		R	G271A	S		S	S543R
227	S		R	A272G (57)	S		S	V16I, S543R
236	S		S		S		S	
239	R	A2143G	R	G271A	R	nd	S	S543R
243	S		S		R	ins121GAAATC-GCC (present study)	S	V16I, S543R
263	R	A2142G (26)	S		S		S	T556S (65), S543R
283	S		R	G271A	S		S	S543R
288	S		S		S		S	

AMX Amoxicillin; CIP Ciprofloxacin; CLR Clarithromycin; LVX Levofloxacin; MTZ Metronidazole; nd Did not detect any mutations that have been previously linked to antimicrobial resistance; R Resistant; rRNA Ribosomal RNA; S Susceptible; TET Tetracycline

have been associated with AMX resistance; however, all of the isolates were susceptible to the antibiotic and, thus, the mutations may not be involved in resistance. They include individual and combinations of V16I, V45I, T556S, A599T, S543R and G595S mutations. Interestingly, some of the isolates have newly found mutations for S543 to asparagine or threonine, or insertions at G595, but whether they appear as single or multiple mutations, they do not appear to be sufficient to confer resistance. No known mutations of 16S rRNA that are responsible for TET resistance were found, which was consistent with the present findings that all of the isolates were susceptible to TET.

DISCUSSION

Monitoring emerging antimicrobial-resistant strains of *H pylori* is paramount for therapeutic treatment to be successful. While Canada has a lower prevalence rate than the global average, increased international travel and immigration from countries that have high endemic rates of *H pylori* should be a signal that surveillance should not only continue in Canada and the world, but also perhaps increase.

The present study was the first to examine antimicrobial susceptibility in Northern Ontario. It is surprising that a relatively high percentage of

resistance to certain antibiotics was found by E-test, namely MTZ (35%), CLR (40%) and CIP/LVX (30%), despite the small number of *H pylori* isolates in the study. To compare, in 2000, Fallone (29) determined that MTZ resistance in Canada was between 11% and 48%, while resistance to CLR was 0% to 12%. The variation in data evaluated by Fallone reflected the difficulty in discerning primary and secondary resistance when results were reported. For CIP/LVX, it was estimated that resistance in Canada was <10% in 2004 (30). It is possible that the data we obtained could be attributed to region-specific demographics, and that a broader and larger sample size would be a better reflection of antimicrobial resistance in Ontario. However, the fact remains that a large percentage of antibiotic resistance was found. The data suggest that not only will physicians need to identify *H pylori* quickly, but also that susceptibility testing should be part of the clinical evaluation so that treatment can be tailored to combat the infection more effectively, rather than relying on consensus guidelines, and to prevent or slow the development of antimicrobial resistance.

In Canada, the incidence of AMX resistance was <2% as of 1997 (48), while there has yet to be any public information on resistance rates, if any, of TET since 2000 (29). Our work is consistent with

previous data in that AMX and TET resistance have yet to become more prevalent in Canada and that, for the time being, they remain effective in *H pylori* eradication.

There was 100% agreement in results determining susceptibility or resistance of *H pylori* between the use of antimicrobial disks and the E-test for five of the six antibiotics tested. For MTZ, 19 of 20 isolates were consistent with one another. This is in relative agreement with previous studies that used conditions closely resembling our methodology, in which there was a 7% discrepancy when evaluating MTZ resistance (49).

While 100% agreement between the E-test and disk diffusion assay was not observed with MTZ, the small percentage of discrepancy should be an indication that outside of MTZ, disk diffusion may be able to provide rapid and cost-effective insight into antimicrobial resistance. The greatest challenge in using it to evaluate MTZ resistance is that a standard zone diameter does not exist and that several other factors complicate a proper evaluation (50). The Clinical and Laboratory Standards Institute, EUCAST and the British Society for Antimicrobial Chemotherapy do not recommend the use of antibiotic disks for clinical studies because the stability of the antibiotics on the disk may be compromised after 48 h to 72 h of growth, which is normally required for testing *H pylori*. Regardless, many laboratories still use this approach as a cost-effective measure to monitor emerging resistant *H pylori* strains. The agar dilution method is considered to be the gold standard and is effective in large batch studies to provide accurate MICs, but is technically too demanding to be a viable test for everyday practice (51,52). The E-test method generally has a more consistent antibiotic release, is able to tolerate prolonged incubation and can provide MICs (53). However, an E-test strip costs approximately 100 times more than an antimicrobial disk, which can be prohibitive in terms of cost and access in developing countries where they try to establish surveillance programs, especially because incidence rates of *H pylori* are often higher in some developing nations (7). As such, laboratories in countries such as India, Iran and Turkey continue to use disk diffusion to determine susceptible and resistant *H pylori* strains, while efficiently selecting for more effective therapies (39,54-56). If the cost and the ease of evaluation are prohibitive factors in screening for *H pylori* antimicrobial resistance, disk diffusion, with the possible exception of MTZ, should be an acceptable approach through the development of standardized zones of inhibition.

The mutations detected in the 23S rRNA gene that led to CLR resistance in the present study were consistent with the same mutations that have been described previously (26). The A2142 and A2143 nucleotides are located in the peptidyl transferase loop of the secondary structure of the gene and, as such, the mutations to guanine cause a conformational change in the loop, leading to decreased binding of CLR (26). Similarly, mutations in the *gyrA* gene (G271A, A272G, which correspond to amino acid changes D91N and D91G) that led to resistance to CIP and LVX are also well known in a previous study (57). These mutations have been very consistent in literature as mutations that confer resistance, and served as a validation for our antimicrobial susceptibility results. Recently, it has been suggested that an efflux pathway may also be involved in CLR resistance (13).

In contrast to CLR and the quinolones tested, the mechanism behind MTZ resistance is significantly more complicated. All seven isolates resistant to MTZ, as determined by E-test, did not possess any mutations that are known to cause *H pylori* to be resistant to the drug (37,58). In fact, it has not been possible to clearly identify a set of *rdxA* mutations that consistently accounted for resistance (59,60). However, two of the strains, those from patient 195 (C16T mutation leading to a stop codon) and patient 243 (an insert of nine nucleotides), did show new mutations that may lead to resistance. These mutations would need to be investigated individually using a control strain to determine whether they are involved with MTZ susceptibility. Studies have shown that other genes, such as *frxA*, may also be involved with MTZ resistance when mutated (61). It has also been shown that MTZ resistance can occur without any mutations to the *rdxA* or *frxA* genes,

suggesting that another mechanism may be involved (62), such as the Fur protein that, when mutated, may be able to eliminate the drug (63). Clearly, the complex mechanism behind MTZ resistance will need to be evaluated in future studies.

In our study, every isolate was susceptible to AMX; however, several mutations that were previously described as being linked to resistance were discovered to be present in our isolates. Amino acid mutations such as V45I and V16I, found in six of our isolates, confirmed previous studies in which they were determined to have no effect on antimicrobial susceptibility (64). The mutations G595S and A599T have also been described previously, although it is unknown what their contribution is to resistance because they were part of a series of mutations that led to drug resistance (64). In the present study, we found amino acid insertions at position 595, yet no resistance was found. Another mutation (T556S) was found to be singly capable of conferring resistance in one study (65); however, this was not the case in the present study. Mutations at serine 543 have also been linked to resistance, although not necessarily as a single mutation (66). Fifteen of the 20 isolates had mutations at this amino acid, but no resistance was conferred. *H pylori* from participant 263 had both T556S and S543R mutations, yet this strain was still susceptible to AMX. This suggests that multiple mutations to *pbp1A* may be required to confer resistance. High-level resistance to AMX may also involve mutations to porin proteins, notably HopB (AlpB), HopC (AlpA) and HopD (65,67).

While we report relatively high resistance rates against CLR, MTZ and CIP/LVX, it should be noted that we were unable to determine whether these were primary or secondary resistances. When treatment with an antibiotic fails, it would normally result in a higher probability of a selection of an isolate that is resistant, particularly with MTZ and CIP and, thus, we cannot preclude the possibility that patients may have been previously treated with antibiotics (68,69). It is then possible that there is horizontal transfer of resistance to susceptible strains in individuals who are infected with more than one strain (70).

H pylori is pathogenic and potentially carcinogenic. The present pilot study provides a small, yet important, glimpse into the prevalence of *H pylori* in Northeastern Ontario, in addition to the frequency and molecular mechanisms of antimicrobial-resistant strains. While the present study examined cases of *H pylori* from one institution within a relatively small geographical area, the high proportion of antimicrobial resistance relative to previous reports is alarming. With the presence of strains in the present study that are resistant to multiple antibiotics, particularly MTZ and CLR, which are most frequently used under current treatment guidelines, carefully devised regimens examining antimicrobial susceptibility of *H pylori* strains and consideration of past use of antibiotics, rather than adherence to consensus guidelines, may be preferable. The Maastricht IV guidelines do allow for appropriate changes in treatment if it is not effective due to high prevalence of antibiotic resistance. However, this information needs to be available before changes can be initiated. Routine antimicrobial susceptibility testing would be a good place to start. Future studies to determine whether our results are found in other jurisdictions will need to be conducted to determine whether increased antimicrobial resistance of *H pylori* is widespread.

Despite emerging studies of other mechanisms of antibiotic resistance, chromosomal mutations in *H pylori* remain the primary source. If other mechanisms are involved, whole genome sequencing provides an opportunity to examine mutations during disease progression or selection, which may lead to a tailored treatment that would facilitate *H pylori* eradication instead of resistance selection.

ACKNOWLEDGEMENTS: This work was funded by the Northern Ontario Academic Medicine Association awarded to FDM. The authors thank Drs Kim Stephen Tilbe, Heather Boudreau, Jagan Reddy and Robert Smith for volunteering their services in the endoscopy unit at HSN and extracting gastric biopsies.

DISCLOSURES: The authors have no conflicts of interest to declare.

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