Phenotypic and Genotypic Antibiotic Resistance Patterns in *Helicobacter pylori* Strains From Ethnically Diverse Population in México

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*Helicobacter pylori* strains carry a range of mutations in genes that confer antimicrobial resistance and restrict the available options to treat the infection. Latin America is a region that conserve a large number of indigenous communities relatively isolated that practice a traditional medicine without consumption of drugs. We hypothesized that rates of antibiotic resistance are lower in these communities. Recent progress in whole-genome sequencing has allowed the study of drug susceptibility by searching for the known mutations associated with antibiotic resistance. The aim of this work was to study trends of antibiotic resistance over a 20-year period in Mexican *H. pylori* strains and to compare susceptibility between strains from Mexican mestizos and from indigenous population; we also aimed to learn the prevalence of mutational patterns in genes gyrA, gyrB, rdxA, frxA, rpsU, omp11, dppA, and 23S rRNA and its association with phenotypic tests. Resistance to clarithromycin, metronidazole, amoxicillin and levofloxacin was determined in 167 *H. pylori* isolates by E-test, and the occurrence of mutational patterns in specific genes was determined by whole genome sequencing (WGS). The trend of resistance over 20 years in mestizo isolates showed significant resistant increase for clarithromycin and levofloxacin to frequencies that banned its clinical use. Resistance in *H. pylori* isolates of native communities was lower for all antibiotics tested. Phenotypic resistance showed good to moderate correlation with genotypic tests. Genetic methods for characterizing antibiotic resistance require further validation in each population.

**Keywords:** *Helicobacter pylori*, antibiotic resistance, whole genome sequencing, phenotype, genotype, indigenous communities
INTRODUCTION

Drug resistance is recognized as one of the major threats to worldwide public health (Laxminarayan et al., 2013). The problem is a highly pressing issue because of the rapid spread of multi-drug resistant bacteria, some of which are no longer treatable with the available antibiotics (Dadgostar, 2019). Antibiotic resistance is the main risk factor for failure to respond to treatment of H. pylori infection (Alfizah et al., 2014). H. pylori is an ancient microorganism that has co-evolved with man for at least 60,000 years (Moodley et al., 2012); still, it represents a significant public health issue since it is the main risk factor for gastric cancer, which is the third cause of death because of all cancers (Hooi et al., 2017). It is estimated that over 90% of all cases of stomach cancer are attributed to H. pylori (Moss, 2017). Eradication treatment against H. pylori may halt the progression of chronic atrophic gastritis and intestinal metaplasia, preventing the development of gastric cancer (Mera et al., 2005; Fuccio et al., 2009).

H. pylori is a small, curved, motile, Gram-negative bacillus that colonizes the stomach mucosa of more than 50% of the world population, particularly in developing countries of Africa, Latin America, the Caribbean and Asia (Sjomina et al., 2018). In contrast, the lowest prevalence is reported in Northern America and Oceania, whereas it is declining in Japan (Leja et al., 2016).

Although there is no global recommendation on the regimen for eradication of H. pylori, there are regional consensus (e.g., European, Latin American, Asiatic) (Rollan et al., 2014; Malfertheiner et al., 2017; Sheu et al., 2017) that in the last decades have recommended mostly a "standard" triple therapy (proton pump inhibitor + clarithromycin + amoxicillin) as the first choice of treatment. However, during the last years, most of these regions have reconsidered their recommendations, because of the increase in resistance to clarithromycin (Thung et al., 2016). Current recommendations vary among regions because susceptibility to drugs is different in each geographic area, but also because of other factors like availability and cost of the drugs. The most recently recommended drugs for H. pylori eradication include fluoroquinolones and rifamycin (Goderska et al., 2018).

The H. pylori strains may carry a range of mutations in genes that confer antimicrobial resistance and limit the available options to treat H. pylori infection. Resistance to clarithromycin is commonly associated to three point mutations in the 23S ribosomal RNA (rRNA) gene, A2143G (69.8%), A2142G (11.7%), and A2142C (2.6%) (Thung et al., 2016); whereas other less frequent mutations include A2115G, G2141A, A2144T, and T2289C. Mutations C2694A and T2717C have been associated with low resistance levels (Marques et al., 2020). Other point mutations have been described in the literature (A1821G, G1826A, and T1830C, C2245T, G2224A, but their role in clarithromycin resistance requires confirmation (Rimbar et al., 2007; Alarcon-Millan et al., 2016; Hashemi et al., 2019). These mutations prevent the interaction of the macrolide with the 23S rRNA. The prevalence of H. pylori resistance to clarithromycin varies among regions. Reported resistance in West/central and South Europe is >20%, and in North Europe it is <10% (Megraud et al., 2013); whereas in strains from Latin American countries the overall prevalence of resistance is 12%, although frequencies ranged from 0% to 60% (Camargo et al., 2015).

Reported H. pylori resistance to metronidazole is 33.1% in Europe and 40% in the USA, whereas higher rates have been reported in developing countries, with frequencies as high as 80% (Rollan et al., 2014; Thung et al., 2016); still, metronidazole is frequently used in eradication regimens (Fiorini et al., 2018). Mutations in the rdxA gene have been identified as the main cause of resistance to this drug (Goodwin et al., 1998). Although it has been found that mutations in other genes could also influence resistance to metronidazole, including frxA, mdaB, omp11, ddpA or rpsU genes, and genes involved in transcription regulation of rdxA or in overexpression of hfa efflux pump; although additional studies are required to clearly demonstrate their association with metronidazole resistance (Alba et al., 2017; Saranathan et al., 2020). H. pylori resistance to amoxicillin has remained low worldwide, with reported frequencies of <2% in European countries (Thung et al., 2016) and 6.6% in Bangladesh (Nishizawa and Suzuki, 2014). Amoxicillin binds to penicillin binding proteins (PPB) interfering with peptidoglycan synthesis causing lysis of replicating bacteria and resistance is mainly associated with mutations in the pBP genes (Nishizawa and Suzuki, 2014). Resistance to fluoroquinolones has been determined in a few countries, with rates of 3.3% in France, 34.5% in China, 31.9% in the USA (Thung et al., 2016) and 40% in Venezuela (Lopez Gasca et al., 2018). Levofoxacin is a 3rd generation fluoroquinolone currently suggested for H. pylori eradication (Malfertheiner et al., 2017); it inhibits DNA gyrase and topoisomerase and mutations in the gyrA gene are associated with resistance (Nishizawa and Suzuki, 2014).

Rates of antibiotic resistance may also vary within countries, where differences in drug exposure may be observed within communities. Latin America is a region that conserve a large number of indigenous communities that have remained relatively isolated and often practice a traditional medicine without consumption of drugs. We hypothesized that rates of antibiotic resistance are lower within these communities. In Mexico, approximately 7% of the Mexican population (over 7 million Mexicans) speak a native language. These ethnic groups have different economic development and lifestyles with little access to medical services (Romero-Hidalgo et al., 2017).

Recent technical progress in whole-genome sequencing has allowed the study of drug susceptibility by searching for the known mutations associated with resistance; this approach may led to the discovery of novel mutations responsible for antibiotic resistance. The aim of this work was to compare the susceptibility to antibiotics in H. pylori strains isolated in a mestizo community versus isolates from indigenous populations. We also aimed to evaluate the performance of resistance detection by genome sequencing as compared with the phenotypic detection, measuring susceptibility in agar plates.
MATERIALS AND METHODS

Bacterial Strains
A total of 167 H. pylori strains isolated during the period from 1997 to 2017 were studied, 111 strains were isolated from biopsies of Mexican mestizo adults with non-atrophic gastritis, intestinal metaplasia, gastric cancer, and duodenal ulcer. Biopsy specimens were cultured as previously described (Avilés-Jiménez et al., 2012). All patients were seen at the Centro Médico Nacional Siglo XXI, Instituto Mexicano del Seguro Social in Mexico City. The study was approved by the ethical committee from the Instituto Mexicano del Seguro Social. In addition, 56 H. pylori strains previously isolated from Mexican indigenous people were included (Camorlinga-Ponce et al., 2011). All H. pylori organisms were stored at −70°C until tested.

Phenotypic Characterization of Antimicrobial Susceptibility
The antimicrobial susceptibility of H. pylori to amoxicillin, clarithromycin, levofloxacin, and metronidazole was determined using the Epsilometer test (E test) (Torres et al., 2001), a test that whereas is not considered as the reference methods, it is commonly used for a number of antibiotics in different bacteria. A recent study reported a good agreement for levofloxacin, clarithromycin, and metronidazole when compared with the agar dilution method, considered as the gold standard (Miftahussurur et al., 2020). H. pylori isolates were grown for 2 days on Columbia blood agar plates (Becton Dickinson, New Jersey, USA) and growth suspended in Columbia broth to achieve a McFarland opacity of 3. Bacterial suspension was spread on Muller Hinton agar medium with 5% of sheep blood (Becton Dickinson, New Jersey, USA) and the E-test strips (Liofilchem, Roseto degli Abruzzi, Italy) were placed on the plate and incubated at 37°C for 72 h with a 10% CO2 atmosphere (Nuairé, Plymouth, Minn). The MIC was defined by the point of intersection of the inhibitory zone with the strip. MIC values were defined according to the clinical breakpoints proposed in the sixth version of the EUCAST (European Committee on Antimicrobial Susceptibility Testing, EUCAST, 9th version, 2019) for H. pylori. The cutoff values for resistance were >8 mg/L for metronidazole, >0.125 mg/L for amoxicillin, >1 mg/L for levofloxacin, and >0.5 mg/L for clarithromycin. Two reference strains were used as controls, H. pylori ATCC 43504 with MICs for clarithromycin, amoxicillin, metronidazole, and levofloxacin of 0.016, 0.016, 64, and 0.064 mg/L, respectively and strain H. pylori 26695 with MICs for clarithromycin, amoxicillin, metronidazole, and levofloxacin of 0.98, 0.94, 0.75, and 0.32 mg/L, respectively.

DNA Extraction and Sequencing
Genomic DNA was extracted from 93 H. pylori strains using the DNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Nine isolates were sequenced with the HiSeq 2000 platform (Illumina, San Diego, CA, USA) using the paired-end method, as previously described (Muñoz-Ramirez et al., 2017). The remaining isolates were sequenced at the Weimer laboratory at the University of California, Davis, (USA) within the 100K Pathogen Genome Project (Weimer, 2017). Isolates (65) were sequenced using Illumina HiSeq2500 (Weis et al., 2016; Draper et al., 2017). While 19 were done using Pacific Biosciences as previously described (Kong et al., 2014). PacBio reads were processed with the SMRT Analysis package (version 1.3). Genomes were assembled de novo by using the hierarchical genome assembly process (HGAP) and polished using Quiver to obtain final consensus assemblies. For the Illumina paired-end 150 reads, we tested k-mers lengths from 31 to 121 using the VelvetOptimiser script (version 2.2.4) (Zerbino and Birney, 2008). The k-mer length that produced the best assembly according to the N50 contig was used to generate the final assemblies using ABYSS (version 2.0.2) (Simpson et al., 2009) (Supplementary Table 1).

Identification of Mutations in Resistance-Associated Genes
Genomic variations were identified in genes conferring antibiotic resistance: 23S rRNA for clarithromycin; rdxA, frxA, rpsU, omp11, and dppA for metronidazole; pbp1, pbp2, pbp3 for amoxicillin; and gyrA and gyrB for levofloxacin. First, open reading frames (ORFs) were predicted from de novo assemblies by a genomic annotation using the Prokka software v1.12 (Seemann, 2014) and the proteome of the 26695 strain (NC_000915) as primary source. Then, genomic coordinates of ORFs were used to extract the entire sequence of target genes. Sequences were then aligned according to genes of the 26695 reference strain (GeneBank accession number U27270.1) using muscle v3.8.31 (Edgar, 2004) and SNPs were called from alignments using SNIPPY (v.4.6.0) (Page et al., 2016) (https://github.com/tseemann/snippy) following the parameters: minimum coverage 20X, a minimum base quality score of 30 and a proportion for variant evidence of 0.9. Mutations were considered according to SNIPPY outputs and visualized using Artemis v18.1.0 (Carver et al., 2012) or MEGAS’s sequence editor (Tamura et al., 2013).

Statistical Analysis
The sociodemographic variables were analyzed as follows, gender and clinical diagnosis were compared with the Kruskal-Wallis test. To compare frequency of resistance to antibiotics between the different periods of isolation the $X^2$ linear trend was used. Frequency of resistance to one or more antibiotics among the study populations was analyzed by means of $X^2$; ORs (Odds Ratio) and their 95% confidence intervals were also estimated. In all cases, a p-value <0.05 was considered as statistically significant.

RESULTS
Characteristics of the H. pylori Strains Studied
Table 1 describes the characteristics of the patients from whom H. pylori strains were isolated. A total of 167 H. pylori strains were included in this study, of these, 111 were isolated from Mexican mestizos (mean age, 48.7 ± 14.2 years; 34 males and 77
females). The clinical diagnosis in these patients were chronic gastritis, pre-neoplastic lesions, gastric cancer and duodenal ulcer. 56 H. pylori strains from Mexican indigenous people were also studied, all individuals with chronic gastritis (mean age 41.63 ± 20.0 years; 21 males and 35 females).

**Antimicrobial Susceptibility**

Analysis of the antimicrobial susceptibility of the 167 isolates revealed high resistance for metronidazole (58.6%), and moderate to levofloxacin (18.5%) and clarithromycin (8.9%), while only 1.8% were resistant to amoxicillin. We next analyzed the trend of resistance over a 20-year period for the mestizo isolates (Figure 1 and Table 2) and found a constant increase from 1997 to 2017 for clarithromycin (1.85%–32.2%, p=0.000) and levofloxacin (9.2%–58.1%, p=0.000). In contrast, resistance to metronidazole tended to decrease (73%–51.6%); whereas for amoxicillin resistance was not detected during 1997–2011 but was already present in 6.5% of the 2017 isolates.

Compared with the mestizo strains, resistance among the isolates of native communities was lower for metronidazole (39.3%), very low for clarithromycin and amoxicillin (1.8% for both) and absent for levofloxacin (0%) (Table 2). Among all the 167 H. pylori strains tested, 30.5% were susceptible to all four antibiotics tested, which corresponded to 15.3% of the mestizos and 66% of the indigenous communities (Table 3). Among all studied strains 56% were resistant to at least one antibiotic and interestingly, 65% of the mestizo vs. 37% of the indigenous strains presented this pattern. Finally, multidrug resistance was also higher in mestizo (18.9%) than in indigenous strains (1.7%) (Table 3). Among the multi-resistant mestizo strains, dual resistance to metronidazole/levofloxacin was more frequently observed (16.2%), followed by resistance to clarithromycin/metronidazole/levofloxacin in 4.5% of the tested strains. Resistance to both metronidazole/levofloxacin rose from 7.4% in 1997 to 1999 to 32% in 2017. A high resistance rate of 25.8% was detected for clarithromycin/metronidazole among H. pylori isolates from mestizos. No strain was resistant to all four antibiotics tested. Of note, among the strains of indigenous origin, only one isolate presented double resistance (clarithromycin/metronidazole).

**Mutation Analysis in the Resistance Genes**

Of the 167 strains included in the study, 93 strains (74 mestizos and 19 indigenous) were whole-genome sequenced, 84 in this study and 9 reported previously (Muñoz-Ramírez et al., 2017) (Supplementary Table 1). Seven of these 93 strains were phenotypically resistant to clarithromycin; of these, one presented the A2146G (formerly A2142G) amino acid variant and four presented the variant A2147G (formerly A2143G) (Table 4); whereas among the 86 sensitive strains two presented variants reported associated with resistance. In this analysis we checked for mutations in the two copies of the 23S rRNA gene.

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**TABLE 1 |** Characteristics of patients from whom H. pylori was isolated in Mexico during 1997–2017.

| Time Period | Total | Mestizo population | Native community |
|-------------|-------|---------------------|------------------|
|             | 1997–2017 | 1997–2017 | 2002–2004 |
| No of strains | n=167 | n=111 | n=56 |
| Age, mean(SD) | 50.72 (14.27) | 48.7(14.2) | 41.63 (20.03) |
| Gender | | | |
| Male, n(%) | 56 (33.6) | 41 (36.9) | 15 (26) |
| Female, n(%) | 111 (66.4) | 70 (63.1) | 41 (74) |
| Diagnosis | | | |
| Gastritis | 119 | 73 | 46 |
| Pre-neoplastic lesions | 18 | 12 | 6 |
| Gastric cancer | 23 | 15 | 8 |
| Duodenal ulcer | 7 | 3 | 4 |

**TABLE 2 |** Antimicrobial resistance of H. pylori strains isolated from Mexican mestizos over a period of 20 years and from native populations.

| Years | aClarithromycin No (%) | bMetronidazole No (%) | Amoxicillin No (%) | cLevofloxacin No (%) |
|-------|------------------------|-----------------------|--------------------|----------------------|
| Mestizo population n=111 | | | | |
| 1997–1999 (n=54) | 1(1.8) | 41(75.9) | 0.0 | 5(9.2) |
| 2006–2011 (n=26) | 3(11.5) | 19(73.0) | 0.0 | 8(30.8) |
| 2017 (n=31) | 10(32.2) | 16(51.6) | 2(6.5) | 18(58.1) |
| Native community | | | | |
| 2002–2004 (n=56) | *1 (1.8) | **22 (39.3) | ***1 (1.8) | 0.0 |
| Total N=167 | 15(8.9) | 98(58.6) | 3(1.8) | 31(18.5) |

X² for linear trend = 15.87, p = 0.0007; bX2 for linear trend = 4.89, p = 0.026; cX2 for linear trend = 23.15; p = 0.0000. X² = 5.33, p = 0.02, OR = 7.93 (1.01 – 62.0) mestizo vs native populations; **X2 = 13.07, p = 0.0002, OR = 3.35 (1.72 – 6.55) mestizo vs native populations; ***Fisher Exact = 0.7, OR = 1.00 (0.89 – 1.13) mestizo vs native populations.
rRNA operon present in *H. pylori*. For levofl oxacin, among the 93 strains 22 were resistant and 17 presented amino acid variants in position 87 and 91 of GyrA (Table 4); however, 14 of the 71 sensitive also presented these variants. The most common variant found in GyrA was N87I, whereas amino acid variants in GyrB were in two resistant strains but present in four of the 71 sensitive isolates.

Regarding metronidazole, there are no reports on mutations clearly associated with resistance, accordingly, we describe all the observed mutations. Mutations in *rpsU* leading to variants A11T and G9S were found in resistant strains (Table 4); the A11T mutants had a MIC >256 to metronidazole. Two isolates sensitive to metronidazole presented the mutation R36G in *rpsU*. In addition, we detected mutations in *omp11*, but the significance of this finding is unclear because mutations were found in both, strains resistant and sensitive to metronidazole (Table 4). In gene *dppA* mutations in I485L, Q382E, and 37del were present only in strains resistant to metronidazole.

A considerable number of mutations was found in *rdxA* and amino acid variants were as much as 64 (Table 5), 32 present only in resistant strains, 22 present in both resistant and sensitive and 10 present only in sensitive strains. Besides the 64 non-synonymous mutations in *rdxA* we also found six frameshifts, three nonsense mutations, 32 missense mutations, and stop codons were observed in resistant isolates in positions 13, 26, 37, 72, 73, and 76. Similarly, in FrxA we found no amino acid variants clearly associated with metronidazole resistance, amino acid variants were present only in three resistant *H. pylori* strains,

### TABLE 3 | Patterns of antibiotic sensitivity of *H. pylori* strains isolated from indigenous and mestizo communities in Mexico.

| Resistance to four antibiotics | Indigenous n=56 (%) | Mestizo n=111 (%) | All n=167 (%) | OR (95% CI) |
|-------------------------------|---------------------|-------------------|---------------|-------------|
| Sensitive                     | 34 (66.6)           | 17 (15.3)         | 51 (30.5)     | 13.6 (6.6–27.7) |
| Resistance to one antibiotic  | 21 (37.5)           | 73 (65.7)         | 94 (56.3)     | 0.31 (0.16–0.61) |
| Resistance To > 2 antibiotics | 1 (1.7)             | 21 (18.9)         | 22 (13.2)     | 0.07 (0.01–0.59) |
| OR, Odds Ratio.               |                     |                   |               |             |

### TABLE 4 | *H. pylori* mutations in genes associated with phenotype resistant to Clarithromycin, Levofl oxacin, and Metronidazole.

|                      | Resistant No (%) | Sensitive No (%) |
|----------------------|------------------|------------------|
| Clarithromycin       |                  |                  |
| A2142G               | 3(30)            | 0                |
| A2143G               | 1(1)             | 1(1)             |
| A2146G               | 1(1)             | 1(1)             |
| A2147G               | 1(1)             | 1(1)             |
| Levofl oxacin        | 32               | 78               |
| GyRA                 |                  |                  |
| N87T                 | 4(12.5)          | 7(8.9)           |
| N87K                 | 6(18.8)          | 3(3.8)           |
| N87I                 | 8(25.0)          | 5(6.4)           |
| T62i                 | 0                | 1(1.2)           |
| D91G                 | 1(3.1)           | 1(1.2)           |
| D91Y                 | 1(3.1)           | 2(2.5)           |
| N87T-D91G            | 1(3.1)           | 0                |
| D91N                 | 1(3.1)           | 4(5.1)           |
| GyRB                 |                  |                  |
| D481E                | 2(2.5)           |                  |
| D481G                | 0                | 1(1.2)           |
| Metronidazole        |                  |                  |
| rpsU                 |                  |                  |
| A11T                 | 3(3.8)           | 0                |
| G9S                  | 3(3.8)           | 0                |
| R36G                 | 0                | 3(9.3)           |
| omp11                |                  |                  |
| V148I                | 12(15.3)         | 12(37.5)         |
| T13A                 | 24(30.7)         | 31(96.8)         |

### TABLE 5 | Amino acid variants present in RdxA of *H. pylori* strains resistant or sensitive to metronidazole.

|                      | Resistant No (%) | Sensitive No (%) |
|----------------------|------------------|------------------|
| 33 amino acid variants present only in resistant *H. pylori* strains | K2R, Q11X, R14K, H17Y, H25R, E27T, 30SR, A37V, S45N, G50R, M56V, T58I, L62S, K63Q, S81L, P106D, P106S, V111A, P115R, A118S, C140R,C140G, S158M, G163D, G163V,K168R, A193V, K168R, A193V, E194G, A206T, I207T |
| 10 amino acid variants present only in sensitive *H. pylori* strains | H53R, A69Y, P96L, V123T, S158G, R176H, D205A, A206E, I207E, T208L |
| 22 amino acid variants present in both sensitive and resistant *H. pylori* strains | Q11H, R16G, R16H, T31E, T32A, M56I, D59N, A67V, R90K, H97Y, H97T, G98S, A118T, S128G, R131K, G170S, V172I, E175Q, A183V, C184R, Q197K, V204I |

### TABLE 6 | Amino acid variants present in FrxA of *H. pylori* strains resistant or sensitive to metronidazole.

|                      | Resistant No (%) | Sensitive No (%) |
|----------------------|------------------|------------------|
| 3 amino acid variants present only in resistant *H. pylori* strains | L52I, A85V, E169A |
| 13 amino acid variants present only in sensitive *H. pylori* strains | V7I, A16T, A32V, I44V, R58G, K60R, V81L, D91N, N110D, N124S, A152V, E169A, E176K |
| 14 amino acid variants present in both sensitive and resistant *H. pylori* strains | L33M, A67T, A70G, L71I; F72S, T110S, N110H, F114Y, M125F, N129T, S130D, E176K, N182D, C193S |
14 were present in both sensitive and resistant strains and 13 present only in sensitive strains (Table 6).

Evaluation of the Performance of Genetic Tests to Detect Antibiotic Resistance

To measure the efficacy of molecular detection of resistance, we estimated the concordance of phenotype vs. genotype using the reported antibiotic resistance associated mutations for clarithromycin and levofloxacin and all found mutations for metronidazole (Table 7). Performance for clarithromycin had a substantial agreement (K = 0.69), which is as good as in reports from other populations (Farzi et al., 2019; Tshibangu-Kabamba et al., 2020); we found two resistant strains with no mutation in 23S, suggesting that in our population other mechanisms are responsible for this phenotype. Thus, 23S genotyping might be recommended for clarithromycin resistance in H. pylori strains from our community. Regarding levofloxacin, genotyping of gyrA and gyrB had a moderate agreement (K = 0.49). Previous studies identified amino acid variants in positions 87 and 91 of GyrA associated with high MIC values for levofloxacin (Lopez Gasca et al., 2018; Hanafiah et al., 2019; Tuan et al., 2019). Genotyping of gyrA might not be an alternative to determine levofloxacin resistance if phenotypic tests are not available. For metronidazole, rdxA and frxA turned out to be highly variable genes with an unusual number of SNPs, and no single mutation clearly associated with resistance resulting in a slight agreement (<0.2); genotyping of rpsU, dppA, rdxA, or frxA is not an option to identify metronidazole resistant strains. Regarding amoxicillin, we found a reduced number of resistant strains and those strains presented no mutations in PBPs. Mutations in resistance-associated genes increased after the period 1997 to 1999 as observed in the periods 2002 to 2011 and 2017 (Table 8), particularly for 23S rRNA, gyrA, and dppA genes.

**DISCUSSION**

In 2017, H. pylori was categorized by the WHO as a bacterium with high antibiotic resistance that poses an important risk to human health (Dang and Graham, 2017). H. pylori antibiotic resistance is the main factor affecting the success of current therapeutic regimens and alternatives for suitable antibiotics to treat this infection are becoming limited. Clarithromycin and amoxicillin are the antibiotics of the standard triple therapy, still in use in many regions for the of eradication H. pylori (Suzuki et al., 2019). However, the resistance to these antimicrobials has increased rapidly during the last decade in several countries (Ogata et al., 2013; Miftahussurur et al., 2016; Park et al., 2016). We aimed to study the pattern of antibiotic resistance in Mexican mestizo population over a period of 20 years. During this period, we found a significant increase in the rate of resistance to clarithromycin and levofloxacin and moderate to amoxicillin; while resistance to metronidazole decreased. Resistance to clarithromycin was low in the first year of the survey, but after 20 years, it increased to levels that banned its clinical use; similar findings have been reported in Taiwan (65%) (Wu et al., 2015), Chile (31.2%) (Gonzalez-Hormazabal et al., 2018) and Brazil (19.5%) (Ogata et al., 2013). Based on our studies, the most recent Mexican consensus no longer recommends the standard triple therapy for H. pylori eradication (Bosques-Padilla et al., 2018).

Over the 20-year study period, the resistance to metronidazole decreased from 75% to 51.6%, a significant reduction that might be related to the fact that its previous widespread use (to treat diarrhea, parasitic infections or gynecological disorders), which favored the selection of resistant strains, has been drastically reduced in the last decades, particularly after the act that commanded written prescription to get antibiotics (Vázquez Tsuji Óscar, 2010). The current 51% rate of resistance is similar to that reported in Alaska (44%), but lower than China (56.6%), Vietnam (69.9 and %), and Colombia (83%) (Ierardi et al., 2013; Camargo et al., 2015).

After failure of the standard triple therapy, the Maastricht IV guidelines recommended levofloxacin as a salvage

**TABLE 7 | Analysis of correlation between antibiotic resistance phenotype and genotype.**

| Antibiotic | Genotype | Phenotype | Kappa |
|------------|----------|-----------|-------|
|            |          | S  R      |       |
| Clarithromycin | S         | 97  3 | 0.67 (0.85–0.91) |
|             | R         | 3   3 |       |
| Levofloxacin  | S         | 53  1 | 0.5 (0.66–0.71) |
|             | R         | 27  29 |       |
| Metronidazole | S         | 32  27 | 0.29 (0.47–0.52) |
|             | R         | 10  32 |       |

**TABLE 8 | Mutational analysis in genes involved with antibiotics resistance of Mexican H. pylori strains isolated over a period of 20 years.**

| Antibiotic | Genotype | 1997-1999 n=39 | 2002-2011 n=40 | 2017 N=31 |
|------------|----------|----------------|----------------|-----------|
| 23S rRNA   |          |                |                |           |
| n=7        |          |                |                |           |
| A2147G     | 1         | (14.2)         | 3 (42.8)       |
| A2147G     | 1         | (14.2)         | 3 (42.8)       |
| Levofloxacin |          |                |                |           |
| n=52       |          |                |                |           |
| GyrA       | N87T     | 0              | (4.5)          | 0         |
| GyrA       | N87K     | 1              | (4.5)          | 3 (13.6)  |
| GyrA       | N87T     | 2 (9)          | (48.1)         |           |
| GyrB       | D91G     | 1              | (4.5)          |           |
| GyrB       | D91Y     | 1              | (4.5)          |           |
| GyrB       | N87T-D91G| 1              | (4.5)          |           |
| GyrB       | D91N     | 2 (9)          | (48.1)         |           |
| Metronidazole | n=59  |                |                |           |
| rpsU       | A11T     | 1 (1.6)        |                | 1 (1.6)   |
| rpsU       | G9S      | 3 (5)          |                |           |
| Omp11      | V148     | 5 (8.4)        | 4 (6.7)        | 2 (3.3)   |
| Omp11      | T13A     | 8 (13.5)       | 9 (15.2)       | 3 (5)     |
| dppA       | 37del    | 1 (1.6)        |                | 3 (5)     |
| dppA       | I465L    | 4 (6.7)        |                |           |
| dppA       | I465V    | 3 (5)          |                | 4 (6.7)   |
| dppA       | A212E    | 12 (20.3)      |                | 8 (13.5)  |
| dppA       | Q238E    | 3 (5)          |                |           |
therapy (Malfertheiner et al., 2017) and other countries even suggested levofloxacin as first line therapy (Heo and Jeon, 2014; Liou et al., 2016). We found that in Mexico, resistance to levofloxacin rise from 9% to 58% in a 20-year period; a 10% resistance has been suggested as the threshold to use levofloxacin as empiric first line therapy (Thung et al., 2016). Levofloxacin was clinically useful at the beginning of the survey, but now it is useless to eradicate *H. pylori* infection in mestizo population. This work is the first report on levofloxacin resistance in *H. pylori* in Mexico, but several works have reported high levofloxacin resistance in Europe and Asia (Megraud et al., 2013; Alflizah et al., 2014; Smith et al., 2014; O’Connor et al., 2015). In Mexico a recent clinical trial compared the utility of triple therapy with clarithromycin versus levofloxacin and found a poor efficacy with both treatments with eradication rates close to 60% (Ladrón-de-Guevara et al., 2019). Quinolones have been widely used to treat urinary tract infections and other infections in Mexico, and the observed high resistance in *H. pylori* strains is most probably the result of exposure when other infections are treated. Studies reporting antibiotic resistance over a period of years are scarce in Latin America, our work clearly show a sharp increase in resistance to clarithromycin and levofloxacin and also the worrying appearance of resistance to amoxicillin. These pronounced changes in susceptibility are an indication of the need to monitor patterns of resistance, particularly in countries with high prevalence of the infection, including the Latin American region (Curado et al., 2019). The resistance rates to clarithromycin, metronidazole, and levofloxacin have increased over time globally, which agrees with studies presenting evidence of an association between antibiotic consumption and antibiotic resistant in *H. pylori* infection (Megraud et al., 2013; Camargo et al., 2015). In Mexico clarithromycin, amoxicillin and metronidazole are antibiotics commonly used to treat other infections, which would also favor this increase in resistance. Therapies including levofloxacin are currently recommended; however, in *H. pylori* isolates from 2017, we found levofloxacin resistance in 58% and multiresistance to metronidazole and levofloxacin of 32%, discarding their clinical use for this infection.

A second aim of this study was to contrast the antimicrobial susceptibility to the most commonly used antibiotics in Mexican *H. pylori* strains isolated from two ethnically different groups. We found that *H. pylori* strains from mestizo population presented higher drug resistance than isolates from native communities. Native communities in Mexico usually follow its cultural customs including traditional medicine where the use of antibiotics is limited (Guzman-Rosas, 2016). It is probably the limited exposure to these drugs the cause of reduced antimicrobial resistance, a positive correlation that not always is observed. There are no reports on the treatment to eliminate *H. pylori* infection in native Mexican people. Our hypothesis was further strengthened with the observation of absence of resistance to levofloxacin an antibiotic that hardly reach these communities. As mentioned above, the most recent Mexican consensus no longer recommend triple therapy (Bosques-Padilla et al., 2018) based on susceptibility patterns in mestizo populations. Our results suggest triple therapy may still be an effective treatment in native communities and call the attention to differences between populations that must be taken into account when working in consensus for regions with both mestizo and native communities. This is regionally important since most Latin American countries still have indigenous populations (Adhikari et al., 2016).

Bacteria use two major genetic strategies to adapt to the exposure of antibiotics. One is mutations in genes associated with mechanism of action of the drugs and the second is incorporation of resistance gene by acquisition of foreign DNA via horizontal transfer (Munir and Arias, 2016; Tuan et al., 2019). Several reports have identified mutations in the genome of *H. pylori* involved in resistance to clarithromycin, fluoroquinolones, and metronidazole. Sequencing of the *H. pylori* genome is increasingly more available and less expensive and offers a unique opportunity to identify known and novel mutations in genes associated with resistance to antibiotics. Experimentally confirmed mutations can then be identified using even simpler and cheaper techniques, some commercially available and designed for use with biopsy tissues without the need to isolate *H. pylori* (Poel et al., 2019; Pichon et al., 2020). However, this is only possible after a thorough confirmation of the specific association with resistance in different regions of the world.

*H. pylori* resistance to clarithromycin is frequently due to point mutation in the 23s rRNA gene and prevalence of these mutations varies geographically even between populations in the same country (Kageyama et al., 2019). In Chinese patients, prevalence of A2143G was 10%–14% (Savoldi et al., 2018; Zhuoqi et al., 2008) and in Malaysian strains it was 90.5% (Alflizah et al., 2014). Furthermore, in Iran A2143G was highly frequent in clarithromycin resistant strains (68.7%) and A2142G was low (5.6%) (Keshavarz et al., 2015); similarly, A2142G was low (9.5%) in *H. pylori* resistant strains from Malaysia (Alflizah et al., 2014), whereas in Brazil A2147G variant was the most common (77.8%) (Martins et al., 2006). We found good correlation between phenotypic resistant to clarithromycin and mutations in 23s rRNA, mainly because the reported mutations were present in resistant strains and mostly absent in sensitive strains. There were however strains resistant to clarithromycin but without mutations in 23s rRNA suggesting the presence of other mechanism of resistance in *H. pylori* in our population.

Resistance to levofloxacin is given by non-synonymous mutations in a region of gyrA or gyrB, determining resistance to quinolones. Amino acid substitutions in GyrA have been described in positions 91 (D91G, N, A, Y, or H) and 87 (N87L, I, A, or K) (Zerbetto De Palma et al., 2017) in addition to other substitutions in GyrB. 87 and 91 substitutions are the more frequently described, suggesting their presence might predict resistance to fluoroquinolones (Zerbetto De Palma et al., 2017; Lopez Gasca et al., 2018). In Colombia an important increase in fluoroquinolone resistance from 2009 to 2014 was reported, mostly associated with mutations in gyrA, with N87I being the most common variant (Wang et al., 2010; Trespalacios et al., 2014).
2015). In our study, we did not find any substitution in GyrA exclusively present in resistant strains and in fact the performance of molecular detection of mutants was moderate in our population, mainly because of the frequent presence of the described resistant-associated variants in sensitive strains. Limiting the test to the detection of variants in position 87 of GyrA increase concordance but sensitivity is still low (detecting in 77% of resistant strains). gyrB mutation (S479G) was identified in two H. pylori resistant strains, indicating that gyrB has no impact on fluoroquinolone resistance in our population. Our results agree with previous reports from Kuala Lumpur and Iran (Hanaﬁah et al., 2019; Tuan et al., 2019).

Resistance to metronidazole involves the inactivation of the rdxA gene that catalyzes the reduction of metronidazole (Tuan et al., 2019). A number of studies have reported amino acid variants in RdxA present only in metronidazole resistant strains (Marais et al., 2003; Butlop et al., 2016). In this study we found amino acid variants in RdxA that were associated exclusively with metronidazole resistant strains in over 50% of the resistant isolates; however, these variants were as much as 32 and none was present in more than 5% of resistant isolates. In addition, 32 other variants were present either in both sensitive and resistant strains or only in sensitive isolates. Thus, it is not a surprise the low concordance found with molecular and phenotypic detection; in addition, considering the large number on mutations in the rdxA gene it seems that molecular detection of resistance is not an option for metronidazole. Furthermore, six metronidazole-resistant strains contained stop codons at position 13, 26, 37, 72, 73, 73; different from those reported previously in positions 2, 50, and 52 (Butlop et al., 2016). We found a number of amino acid variants present only in resistant H. pylori isolates. Substitutions in rpsU (A11T) and dppA (1485L, 37del) were found to be present only in resistant isolates however, the role of these variants in resistance to metronidazole remains to be elucidated because we had a high percentage of resistant strains that did not present any of the studied mutations, whereas other mutations were present in both sensitive and resistant strains. The comparison between the phenotypic and genotypic methods is reported for different authors in various countries with diverse results (De Francesco et al., 2010; Tuan et al., 2019; Mascellino et al., 2020). Mascellino et al, reported good correlation genotype-phenotype among H. pylori resistant to levofloxacin but not in clarithromycin resistant strains (Mascellino et al., 2020). While Tuan et al. (2019) reported excellent genotype to phenotype agreement for clarithromycin and good agreement for levofloxacin and amoxicillin but no agreement for metronidazole.

We have to keep in mind that other mechanisms of metronidazole resistance have been described, including mutations in rfxA, dapF, and efflux proteins (Miftahussurur et al., 2016) (Table 8).

In this study, we showed that in Mexican H. pylori strains there was an important increase in resistance to clarithromycin and levofloxacin and appearance of resistant to amoxicillin during a period of 20 years, which has important clinical implications. We also report a significantly lower frequency of antibiotics resistance in H. pylori strains from native communities. Finally, we found good to moderate performance of genetic test to detect antibiotic resistance; thus, molecular methods for characterizing resistant genes require further validation in each population.

**DATA AVAILABILITY STATEMENT**

The data set analyzed in this work is publicly accessible under the NCI bioproject PRJNA338771 (biosamples: SAMN05569559, SAMN05569561, SAMN05569574, SAMN05569575, SAMN05569581-SAMN05569583, SAMN05569586, SAMN05569587, SAMN05569589-SAMN05569592, SAMN11483334 and SAMN11483336); bioproject PRJNA203445 (biosamples: SAMN09935045-SAMN09935055, SAMN09935057-SAMN09935061, SAMN09935063, SAMN09935064, SAMN10881935, SAMN10881940, SAMN10881942, SAMN10881945-SAMN10881948, SAMN10881953, SAMN10881959, SAMN10881963, SAMN10881965, SAMN10881972) and bioproject PRJNA681870 (biosamples SAMN16970063-SAMN16970092 and SAMN16970108-SAMN16970125) (Supplementary Table 1).

**ETHICS STATEMENT**

The study was approved by the ethical committee from the Instituto Mexicano del Seguro Social.

**AUTHOR CONTRIBUTIONS**

MC-P designed and coordinated the study and wrote the manuscript. JT participated in design and coordination of the study, and wrote the manuscript. AE-O performed the isolation and antimicrobial susceptibility test of H. pylori. AG-D participated in statistical analysis. EA-Z and RT participated in the bioinformatics analysis. SG-C contributed with revising the manuscript. JT participated in statistical analysis. EA-Z and RT participated in the bioinformatics analysis. SG-C contributed with revising the article. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.539115/full#supplementary-material
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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