Genomic determinants of antibiotic resistance for *Helicobacter pylori* treatment: a retrospective phenotypic and genotypic observational study

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Summary

Background Rising antimicrobial resistance of *Helicobacter pylori* is a public health challenge. Genomic-based susceptibility testing allows for the identification of resistance-associated mutations, complementing conventional diagnostics and advancing towards pathogen-based personalised therapies. Our study aimed to identify genes and mutations involved in antimicrobial resistance in *H pylori* and evaluate the extent to which these markers can be used as predictors of phenotypic resistance against clarithromycin and levofloxacin.

Methods In this retrospective phenotypic and genotypic observational study, we included 1011 *H pylori* whole-genome sequences and strains of known geographical origin from the *H pylori* Genome Project (*Hp*GP) collection. We performed phenotypic clarithromycin and levofloxacin susceptibility testing on a subset of 419 *Hp*GP strains using Etest at a centralised laboratory. A genomic analysis was conducted to identify *23S rRNA* and *gyrA* variants and build a curated catalogue of mutations associated with resistance to clarithromycin (ie, *23S rRNA* 2142A→G, 2142A→C, and 2143A→G) and levofloxacin (ie, *gyrA* A88V or A88P, N87K or N87I, and D91G, D91N, or D91Y). Genotype–phenotype concordance was assessed to estimate sensitivity and specificity, and the curated catalogue of resistance-associated mutations was applied to the complete *Hp*GP set. Region-specific prevalence of resistance-associated mutations was calculated for a combined dataset including the *Hp*GP genomes and 768 whole-genome sequences retrieved from the US National Center for Biotechnology Information Sequence Read Archive repository. Associations between resistance genotypes, *H pylori* subpopulations, and minimum inhibitory concentrations (MICs) were tested.

Findings Clarithromycin-resistant and levofloxacin-resistant *Hp*GP strains were estimated with a sensitivity and specificity of 100%, with all confidence intervals ranging from 96% to 100%. The combined analysis (n=1779) found the highest prevalence of clarithromycin resistance in the western Pacific region (173 [51·2%] of 338 in southeast Asia and 75 [29·8%] of 252 in eastern Asia), north African region (seven [38·9%] of 18), and western Asian region (12 [31·6%] of 38), whereas the highest prevalence of levofloxacin resistance was found in south Asia (14 [51·85%] of 27), Central America (48 [38·7%] of 124), eastern Europe (four [36·4%] of 11), and southern Africa (three [33·3%] of nine). Similarly, *23S rRNA* and *gyrA* genotypes are variable across *H pylori* subpopulations. MIC values changed depending on the specific mutation in *23S rRNA* (mean clarithromycin MIC 24·61 mg/L [95% CI 12·27–36·96] for 2143A→G and 142·25 mg/L [95% CI 77·88–206·61] for 2142A→G) and *gyrA* (mean levofloxacin MIC 9·66 mg/L [95% CI 6·75–12·56] for mutations on codon 91, and 27·97 mg/L [95% CI 25·82–30·11] for mutations on codon 87).

Interpretation Mutations in specific genes are reliable indicators to clarithromycin and levofloxacin resistance in *H pylori*, making them useful markers for the development of diagnostic assays and molecular monitoring. Our results suggest that using clarithromycin and levofloxacin empirically, without previous susceptibility testing, is unsuitable in all geographical regions covered by this study.

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Introduction

Eradication treatment of about a quarter of individuals infected with *Helicobacter pylori* is unsuccessful due to resistance-conferring mutations selected during previous treatments for other infections or during the ongoing *H pylori* treatment and due to the increased prevalence of antimicrobial-resistant strains.[1] Treatment of *H pylori* infection commonly consists of a triple therapy based on amoxicillin and clarithromycin with a proton pump inhibitor or bismuth-quadruple therapy.[2] Other first-line

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See Online for appendix 1

Research in context

Evidence before this study

We searched PubMed for publications about genotypic versus phenotypic antimicrobial testing focused on the *23S rRNA* gene in *Helicobacter pylori* using the search terms "*Helicobacter pylori*" AND "resistance" AND "23S" AND ("genotypic drug susceptibility testing" OR "genotype"), obtaining 88 results. The same was done for the *gyrA* gene with the search terms "*Helicobacter pylori*" AND "resistance" AND "*gyrA*" AND ("genotypic drug susceptibility testing" OR "genotype"), obtaining 31 results. For both searches, articles published from Jan 1, 1998, to Jan 10, 2022, were considered without any language restriction. Most publications included gene amplification or sequencing of the *23S rRNA* and *gyrA* genes. A2142 and A2143 mutations were sought in 49 and 70 articles, respectively, linking these variants with clarithromycin resistance. Regarding levofloxacin, mutations at positions N87 and D91 were described in nine and eight articles, respectively, associated with resistance. In most of these studies, the datasets were small and based on gene amplification or limited sequencing of only the genes of interest, instead of using whole-genome sequencing.

Added value of this study

By comparing genotypic versus phenotypic antimicrobial susceptibility testing in 419 *H pylori* strains from the *H pylori* Genome Project (*Hp*GP), we show that resistance to clarithromycin and levofloxacin can be accurately predicted using a catalogue of genomic mutations. Along with a new curated genomic dataset of 1011 *Hp*GP genomes from different settings, we provide a list of validated mutations that explain all the resistance found in the phenotyped part of the dataset for both antibiotics, which contrasts with the often more complex genetic underpinnings of antimicrobial resistance in other pathogens. This study also highlights regional differences in resistance patterns, with novel insights in under-reported areas such as the African continent. In addition, using a catalogue of known mutations for other drugs not tested in vitro, the prevalence of resistance for different eradication regimens was estimated.

Implications of all the available evidence

Together with available evidence, our study supports the implementation of genome sequencing to guide antimicrobial resistance detection in *H pylori*. Validated mutation catalogues allow for accurate resistance prediction for clarithromycin and levofloxacin and could be used to complement phenotypic testing. According to this and previously published studies, resistance prevalence for both antibiotics varies by region, suggesting the need to develop adapted treatment guidelines. In addition, our results also encourage continued surveillance, as new or rare mutations might become clinically relevant in the coming years. These kinds of studies allow the implementation of tailored treatments based on an early evaluation of the resistance profile for clarithromycin and levofloxacin in each case. However, our findings also stress that further research is needed to clearly identify genomic determinants of resistance to other drugs.

treatment combinations also contain metronidazole, tetracycline, levofloxacin, or rifabutin.

The rising rates of treatment failure make the accurate identification of antimicrobial-resistant (AMR) strains increasingly important. Identification of AMR strains has typically been done using phenotypic antimicrobial susceptibility testing (pAST), which requires time-consuming, culture-based methods, specific conditions for biospecimen transportation, selective agar plates, and expertise in culturing this fastidious microorganism.[3]

Genotypic antimicrobial susceptibility testing (gAST) has identified AMR-associated mutations for other infectious pathogens. The WHO *Mycobacterium tuberculosis* mutations catalogue is the most comprehensive example.[4] This catalogue can predict with high sensitivity and specificity AMR to rifampicin and isoniazid, the major drugs in the first-line treatment against tuberculosis. This approach has also proved to be successful for other pathogens such as *Enterococcus faecium*.[5] Such a catalogue does not exist for *H pylori*, although intense work has been done to identify genes and specific AMR-associated mutations,[6,7] mostly focused on specific genes, rather than on genome-wide approaches, or limited to specific geographical regions. Likewise, whole-genome sequencing studies are limited by sample size, geographical extent, or rely on publicly available data to gather phenotypes.[8–12]

See Online for appendix 2

Whether current knowledge of AMR targets and mutations is enough to predict susceptibility to the main drugs used for *H pylori* treatment remains unclear. This hampers the implementation of rapid diagnostic tests, especially relevant for *H pylori* as pAST has significant inter-laboratory inconsistencies. In addition, rapid and accurate detection of AMR can guide treatment choices for infections whose standard of care is often based on empirical treatment rather than being guided by susceptibility profiles,[13] and where prevalence differs across regions.[1] Finally, data are even scarcer for multidrug-resistant strains, which involve phenotypic resistance to more than two of the drugs used in combined therapy and pose an additional risk for prevention and eradication.

Using long-read sequencing data and strains from the *H pylori* Genome Project (*Hp*GP),[14] our study aimed to identify genes and mutations involved in AMR in *H pylori*. In addition, by combining *Hp*GP data with available short-read sequencing data, we aimed to estimate the global prevalence of resistance to commonly used antibiotics.

Methods

Study population and design

In this retrospective phenotypic and genotypic observational study, we included genomic data and *H pylori* strains from the *Hp*GP dataset (n=1011; appendix 2 p 1), which was

further partially phenotyped in this study, and retrieved 768 available whole-genome Illumina sequences (appendix 2 p 2) from the US National Center for Biotechnology Information (NCBI)'s Sequence Read Archive repository (last accessed March 28, 2024). We included samples of known geographical origin in studies focused on *H pylori* infection and disease. Intra-patient samples (ie, collected from the same individuals at different times) were excluded.

As previously described (and expanded in appendix 1 [pp 1–2]),[14] the *Hp*GP consortium generated PacBio de novo genome assemblies of 1011 *H pylori* strains collected between 1995 and 2020 from 50 countries. The *Hp*GP genomes represent four main *H pylori* population clusters, divided into 17 subpopulations. The strains were obtained from individuals with various gastric conditions who provided written informed consent. This study was deemed exempt from US National Institutes of Health (NIH) Institutional Review Board review by the NIH Office of Human Subjects Research Protections because only genome sequences of de-identified bacterial isolates were used.

Procedures

In this study, centralised pAST for clarithromycin and levofloxacin was assessed in 419 *Hp*GP strains in a reference laboratory at the University of Bordeaux (Bordeaux, France). The strains were cultured on an in-house prepared human blood agar supplemented with antibiotics. The media were incubated in a microaerobic atmosphere (85% N_2, 10% CO_2, and 5% O_2) in a special workstation (Baker Ruskinn, Concept Ruskinn, Bridget, UK) at 36°C. After 2 days, the plates were observed every day and colonies were tested for oxidase, catalase, urease, and morphological observation. pAST was performed on Mueller–Hinton agar containing 10% horse blood and globular extract prepared every week, using Etest strips (bioMérieux) for clarithromycin and levofloxacin. The cutoff values used were those recommended by the French Microbiology Society Antibiogram Committee: clarithromycin resistance if the minimum inhibitory concentration (MIC) was greater than 0·5 mg/L and levofloxacin resistance if the MIC was greater than 1 mg/L.[15] All MICs were measured by two independent readers (LP and BL). Quality control was conducted using the *H pylori* strain CCUG 17874 (Culture Collection University of Gothenburg, Gothenburg, Sweden).[16]

Statistical analysis

The sample size was calculated to detect a difference between two independent proportions with a two-sided test, 80% power, and significance level of 0·05. We used three prevalence estimates (ie, 10% for the Americas region, 18% for the European region, and 33% for the eastern Mediterranean region) for primary clarithromycin resistance from the meta-analysis by Savoldi and colleagues.[17] The corresponding sample sizes ranged from 49 to 295. The maximum estimate was inflated by 50% (n=443) to account for difficulties processing the bacterial cultures. The set represents 20 resistance-enriched samples, selected based on partial results provided by international collaborating centres, and 423 samples selected randomly.

Available literature was reviewed for genes associated with resistance to any of the prescribed antibiotics in *H pylori* (appendix 1 p 3). For clarithromycin, the *23S rRNA* gene is widely described as a robust predictor of resistance.[7,18,19] For levofloxacin, mutations in *gyrA*, and to a lesser extent in *gyrB*, have been associated with resistance.[20] The *23S rRNA* and *gyrA* sequences from the 1011 *Hp*GP genomes were extracted with BLASTN v2.5.0, using the *H pylori* reference genes (GenBank accessions: U27270.1:372-3339 for *23S rRNA* and CP079087.1:752290-754773 for *gyrA* and CP079087.1:527460-529781 for *gyrB*). Multiple sequence alignment was used to identify variants. Using the pAST data, we performed a Bonferroni-corrected, one-tailed upper Fisher's exact test to identify mutations positively associated with resistance. Details of the bioinformatic and statistical approach are described in appendix 1 (pp 3–4).

Comparison of gAST versus pAST was performed for the significantly associated mutations to assess their predictive power for clarithromycin and levofloxacin resistance and to determine unexplained resistant cases. In this context, true positive (*TP*) cases are those with a resistant pAST and a resistant gAST, true negative (*TN*) cases are those with a susceptible pAST and a susceptible gAST, false positive (*FP*) cases are those with a susceptible pAST and a resistant gAST, and false negative (*FN*) cases are those with a resistant pAST and a susceptible gAST. Taking this into account, the sensitivity ($\frac{TP}{TP+FN}$) and specificity ($\frac{TN}{TN+FP}$), and the positive predictive values ($\frac{TP}{TP+FP}$) and negative predictive values ($\frac{TN}{TN+FN}$), achieved by every mutation alone and combined by genes were calculated. We also estimated the 95% CI for each value, using the Wilson score interval.

In addition, the prevalence of each mutation in the different global sociodemographic regions (as defined by Comas and colleagues[21]) was calculated by dividing the strains from each region that harbour the mutation by the total strains with the mutation from that region.

We performed association analyses between different genotypes and the *H pylori* subpopulations (as identified by Thorell and colleagues;[14] appendix 2 p 1) using a χ^2 test, and between different genotypes and the MIC levels using a Mann–Whitney *U* test (appendix 1 p 4). We also conducted a multivariate ANOVA to examine the association between genotypes, the *H pylori* subpopulations, and the country of sample origin.

By combining the drug resistance characterisation of the 419 phenotyped *Hp*GP samples and their geographical origin, we estimated continent-wide resistance prevalence for clarithromycin and levofloxacin by calculating the proportion of resistant samples from each continent divided by the total number of resistant samples for each drug in that continent. Additionally, we estimated the trend in resistance prevalence for clarithromycin and levofloxacin between 1995 and 2021 by grouping our dataset in 5-year

For the ***H pylori* strain CCUG 17874** see https://www.ccug.se/strain?id=17874

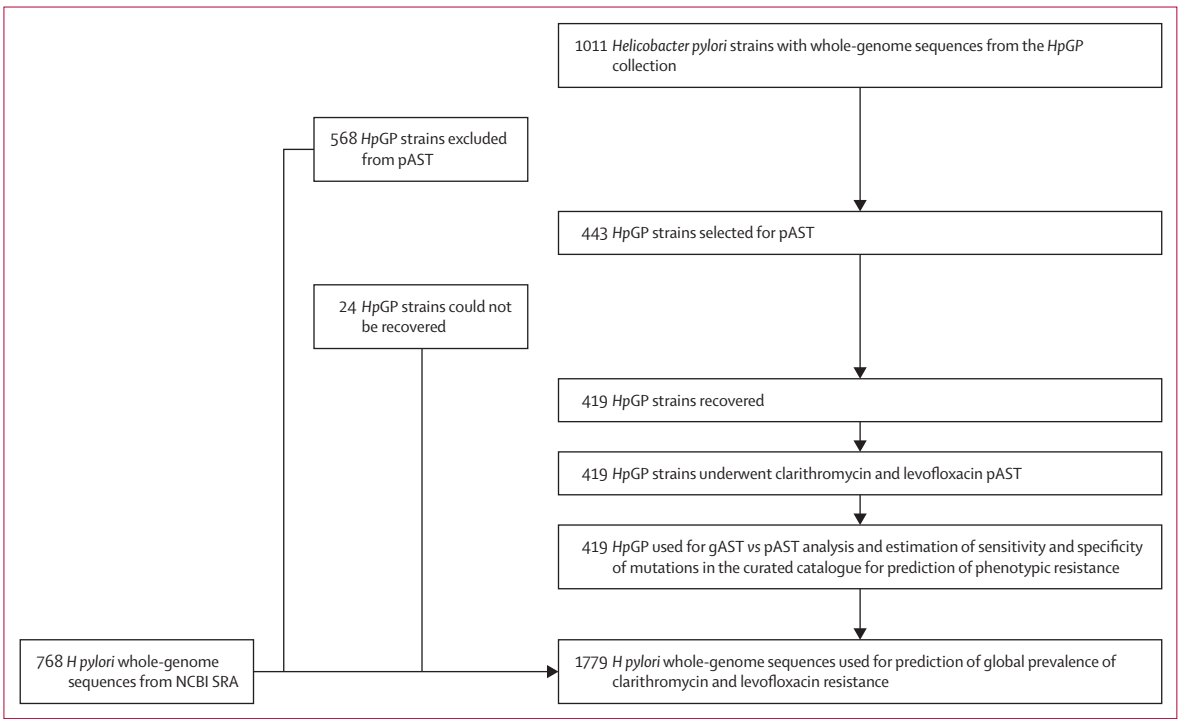


Figure 1: **Study profile**
gAST=genomic antimicrobial susceptibility testing. *Hp*GP=*Helicobacter pylori* Genome Project. pAST=phenotypic antimicrobial susceptibility testing. NCBI SRA=US National Center for Biotechnology Information Sequence Read Archive.

intervals and considering the 419 phenotyped *Hp*GP samples.

We performed an analysis of global, genomic-based prevalence of clarithromycin and levofloxacin resistance by combining the *Hp*GP dataset (n=1011) and the NCBI dataset (n=768), reaching a total of 1779 *H pylori* strains (combined *H pylori* dataset). To assess the presence in the combined *H pylori* dataset of the variants associated with resistance previously defined by gAST or pAST comparison, we extracted *23S rRNA*, *gyrA*, and *gyrB* genes from the samples, made a multiple sequence alignment, and then performed variant calling, as detailed in appendix 1 (pp 3–4). The prevalence of clarithromycin and levofloxacin resistance per sociodemographic region was estimated for both the *Hp*GP and combined *H pylori* datasets by dividing, for each region, the samples harbouring any of the mutations in the catalogue by the total amount of samples from that region. Sociodemographic regions proposed by Comas and colleagues[21] (as shown in appendix 1 p 11) were used to group the prevalence in different countries.

We sought point mutations with strong evidence in the literature of conferring resistance against different rifamycins and tetracyclines, namely *rpoB* L525I, L525P, Q527K, Q527R, D530E, D530G, D530N, D530V, H540L, H540N, H540Y, S545L, L547F, I586L, and I586N,[22–25] and *16S rRNA* 926–928AGA→TTC, 926–928AGA→TGA, 926–928AGA→GGA, and 926–928AGA→GTA,[26–28] respectively. To search for these mutations, the same pipeline as described above was used.

With these results, we estimated the prevalence in the *Hp*GP dataset of resistant strains to at least one of the drugs comprising the following regimens, detailed in appendix 1 (pp 4–5): triple clarithromycin, metronidazole and rifampicin-based therapy, sequential treatment, concomitant therapy, hybrid therapy, reverse hybrid therapy, novel concomitant therapy, bismuth-based therapy containing metronidazole, bismuth-based therapy containing levofloxacin, and levofloxacin-containing therapy. We also searched for double-resistant *H pylori*, defined as a strain resistant to at least two drugs. A $p<0{\cdot}05$ was considered statistically significant for all analyses. Analyses were performed with R version 4.3.2. The packages used are detailed in appendix 1 (pp 3–4).

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Of the 1011 *H pylori* strains included in the *Hp*GP dataset, 443 were selected for pAST (figure 1). Of these, 24 could not be recovered, so we used a subset of 419 strains (appendix 2 p 1) to identify mutations associated with clarithromycin and levofloxacin resistance.

Based on our phenotypic *Hp*GP data, 113 (26·9%) of 419 strains were resistant to clarithromycin and 134 (31·9%) were resistant to levofloxacin. The overall distribution of

these resistant strains varied across geographical regions. For clarithromycin, the highest prevalence was found in Asia (30 [33·3%] of 90) and Europe (59 [32·8%] of 180), whereas the lowest was found in the Americas (20 [15·4%] of 130). For levofloxacin, the highest prevalence was found in the Americas (47 [36·2%] of 130) and Asia (32 [35·6%] of 90), whereas the lowest was in Africa (four [21·0%] of 19). Country-specific estimates are presented in appendix 2 (p 3) and visualised in figure 2.

We found two *23S rRNA* mutations significantly associated with clarithromycin resistance: 2142A→G (A2142G; p<0·0001), present in 16 (14·1%) of 113 clarithromycin-resistant strains, and 2143A→G (A2143G; p<0·0001), present in 98 (86·7%) clarithromycin-resistant strains, with two strains harbouring both mutations, whereas the 2142A→C (A2142C) mutation, also described as associated with resistance, was found in one resistant strain. However, it was not possible to determine an association for this mutation due to its low frequency in the dataset. These three mutations were included in our catalogue. In this study, no significant associations were found with in-vitro macrolide resistance for 2245C→T (C2245T; corrected p>0·99), present in one susceptible strain, and 2182C→T (T2182C; corrected p>0·99), present in 93 strains, both susceptible and resistant.

For levofloxacin, five *gyrA* mutations, N87K (48 [35·8%] of 134, p<0·0001), N87I (25 [18·7%], p<0·0001), D91N (27 [20·1%], p<0·0001), D91G (24 [17·9%], p<0·0001), and D91Y (10 [7·5%], p=0·0020), were significantly associated with phenotypic resistance, with two strains sharing N87I and D91Y mutations. Our study did not find an association with A88V or A88P (p=0·92 in both cases), each of them identified in one strain, despite being consistently described in the literature.[29] Still, we included them in the catalogue since they appeared in resistant strains not explained by the other mutations. A88P appeared in one strain, *Hp*GP-POR-002, whereas A88V was found in *Hp*GP-TWN-007, both of them resistant. No other mutations in *gyrA or gyrB* reached statistical significance.

Using the three *23S rRNA* and the seven *gyrA* mutations included in our catalogue, 113 strains were predicted as resistant to clarithromycin and 134 strains as resistant to levofloxacin. For clarithromycin, sensitivity and specificity were 100% (95% CI 96·7–100) and 100% (95% CI 98·8–100), respectively, and positive and negative predictive values were 100% (95% CI 96·7–100) and 100% (95% CI 98·8–100), respectively. For levofloxacin, sensitivity and specificity were 100% (95% CI 97·2–100) and 100% (95% CI 98·7–100), while positive and negative predictive values were 100% (95% CI 97·2–100) and 100% (95% CI 98·7–100). In both cases, we considered all the candidate mutations (appendix 2 p 4). The mutations with the highest diagnostic value were also identified. In this dataset, the *23S rRNA* mutation 2143A→G contributes to 85·0% (96 of 113) of the total resistance (figure 3).

For levofloxacin, the *gyrA* N87K mutation achieved a sensitivity of 35·8% (95% CI 35·1–36·5), whereas the

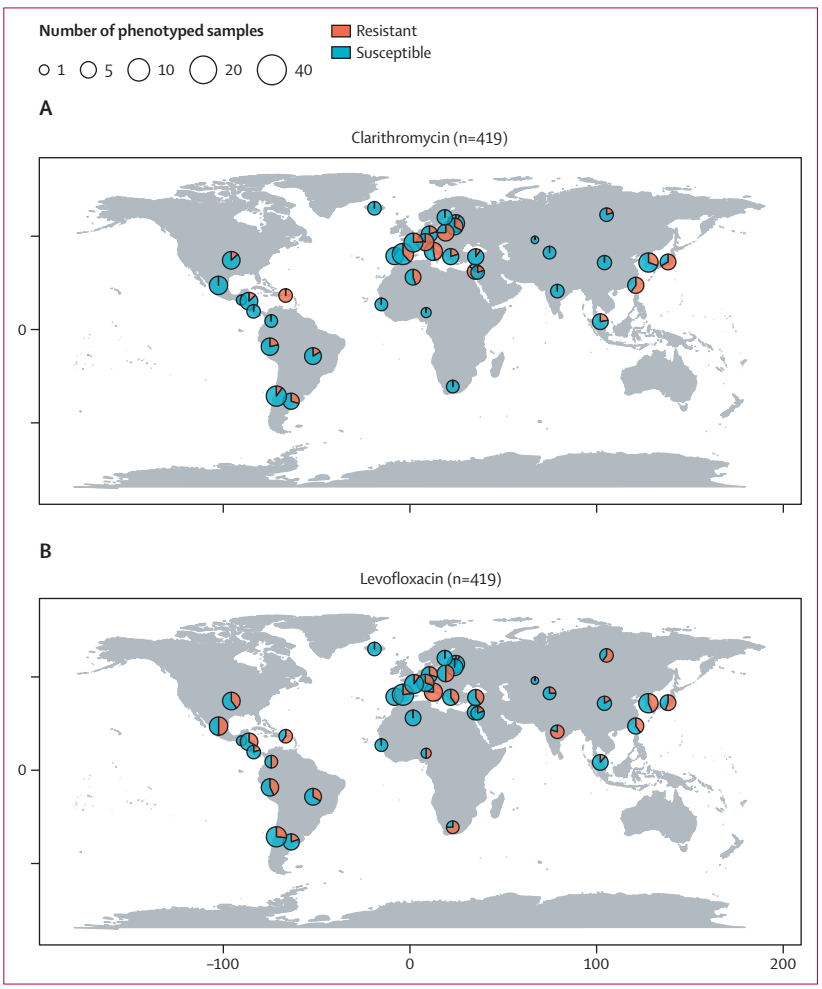


Figure 2: **Global distribution of antimicrobial resistance phenotypes for clarithromycin (A) and levofloxacin (B) in *Hp*GP**
*Hp*GP=*Helicobacter pylori* Genome Project.

remaining mutations gradually increased the overall sensitivity (figure 3). All mutations in the evaluated catalogue had a specificity and a positive predictive value of 100% in our dataset (ie, no false positives were present in our analysis). However, the wide estimated confidence interval might reflect the low prevalence of some mutations. A list of AMR-associated mutations confirmed by this analysis, along with their p-values, is provided in appendix 2 (p 5).

We observed that *23S rRNA* mutations were more prevalent in subpopulations (per Thorell and colleagues[14]) hspSWEurope, hspEasia, and hspEurasia (χ^2 test, p=0·0031, p=0·0057, p=0·0010, respectively) and less prevalent in the hspSWEuropeLatinAmerica (p=0·0003), in both cases comparing against the rest of the subpopulations. However, these results do not hold after accounting for geographical regions using a multivariate ANOVA test. We did not detect any significant association for the *gyrA* variants. These results are expanded in appendix 1 (p 5).

Among the *23S rRNA* mutations, 2142A→G had higher mean clarithromycin MIC values (142·25 mg/L [95% CI

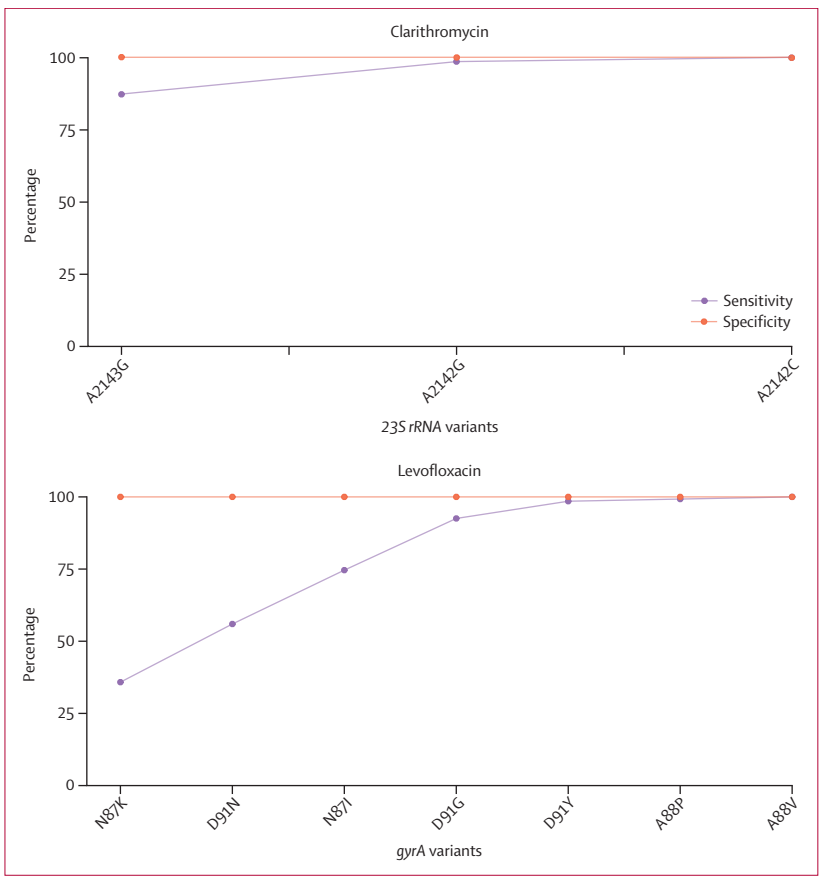


Figure 3: **Sensitivity and specificity values of the genetic determinants explored for clarithromycin and levofloxacin resistance**

The x-axis shows the mutations in our catalogue after literature revision and identification of resistance-associated mutations. The y-axis shows the cumulative percentage value of sensitivity and specificity as we include the mutations.

77·88–206·61]) than 2143A→G (24·61 mg/L [95% CI 12·27–36·96]; Mann–Whitney *U* test p<0·0001; figure 4). Regarding *gyrA*, D91 variants had lower mean MIC values (9·66 mg/L [95% CI 6·75–12·56]) than N87 variants (27·97 mg/L [95% CI 25·82–30·11]; Mann–Whitney *U* test p<0·0001). Variants found in codon N87 showed significant differences (Mann–Whitney *U* test p=0·0010). A88 variants showed similar MICs; however, there was only one representative of each of these genotypes, preventing the application of any statistical test. Finally, the genotypes in which two resistance-associated variants (N87I and D91Y) appeared showed the highest MIC values (>32 mg/L).

We estimated global resistance prevalence for the full *Hp*GP dataset (displayed by region in figure 5A), with an overall prevalence of clarithromycin resistance of 16·1% (163 of 1011) and of levofloxacin resistance of 21·5% (217 of 1011). Focusing on the different sociodemographic regions (figure 5A), a prevalence of clarithromycin resistance greater than or equal to 15% was found in northern Africa and western and eastern Asia, Central Africa, southeast Asia, and eastern Europe. A prevalence of levofloxacin resistance greater than or equal to 15% was found in all regions except for Europe and northern Africa, with the highest values in southern Asia, different regions of Africa, and Central America and South America. We consider 15% as a threshold to define a high incidence of resistance according to the Maastricht VI/Florence consensus.[2] Regarding the evolution of resistance prevalence over time in our dataset, we observed a slow but firm increase between 1995 and 2021 for both clarithromycin and levofloxacin (appendix 1 p 6), going from 0% to about 28·9% in clarithromycin and from about 16·7% to 35·8% in levofloxacin.

419 (23·6%) the 1779 strains of the HPc dataset were predicted as clarithromycin resistant and 333 (18·7%) as levofloxacin resistant (figure 5B); 118 (6·6%) strains were resistant to both antibiotics. Specific mutations found for both the *Hp*GP alone and HPc dataset strains can be found in appendix 2 (pp 1–2). Similar to the *Hp*GP dataset, the prevalence of clarithromycin resistance greater than or equal to 15% was found in eastern Asia, north Africa, western Asia, and Europe (figure 5B). The prevalence of levofloxacin resistance rate was greater than or equal to 15% in all regions except for Europe, North America, northern Africa, and southeast Asia, with the highest values present in southern Asia, Central America, and eastern Europe. We compared our prevalence estimations after grouping by WHO-defined regions (Africa, the Americas, South-East Asia, Europe, Eastern Mediterranean, and Western Pacific) and observed similar values to those described by Savoldi and colleagues.[17]

After screening for mutations with strong evidence of being associated with AMR according to the literature, 37 (3·7%) of 1011 strains had at least one described mutation associated with tetracycline resistance (33 strains with 926–928AGA→GGA and four strains with 926–928AGA→TGA in the *16S rRNA* gene) and 6 (0·6%) of 1011 associated with resistance to rifamycins (two strains with D530N, one strain with D530G, one strain with H540N, one strain with H540L, and one strain with I586L, all in the *rpoB* gene). Altogether, including mutations associated with resistance to clarithromycin and levofloxacin, 356 (35·2%) of 1011 strains harboured one resistance mutation. 69 (6·8%) of 1011 *H pylori* strains in the dataset had double resistance. The gAST resistance profile of each strain and a summary combining all antimicrobials are shown in appendix 2 (pp 17–24).

Other variants described in the literature are frequent in the dataset. For example, more than 513 (50·7%) of 1011 strains harbour the *rdxA* variant T31E, which is proposed as being associated with metronidazole resistance.[30] All the screened variants, which have been associated with AMR, are shown in appendix 2 (pp 11–18).

Considering the antimicrobial families with validated mutations, we calculated the prevalence of resistance in different standard regimens, based on resistance to at least one of the antibiotics present (appendix 2 p 19). For most of the regimens, the complete resistance profile could not be evaluated as it included antimicrobials

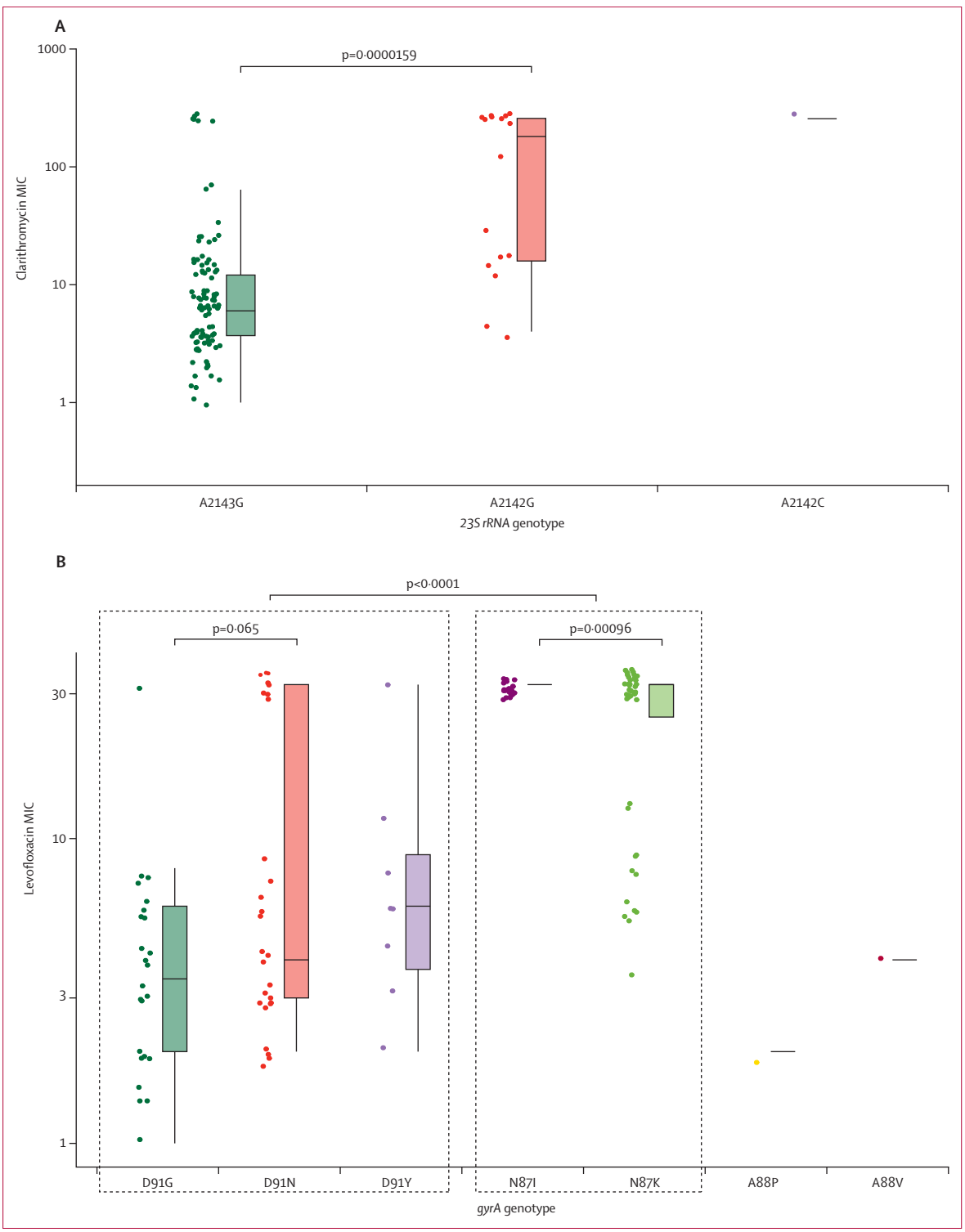


Figure 4: **Association analysis between MIC levels and the different genotypes**
(A) MIC values (mg/L) for each strain containing the described mutations in *23S rRNA*, showing statistically significant differences (Mann–Whitney *U* test) between the MIC values associated with 2143A→G and 2142A→G. MIC datapoints have been jittered in the plot to prevent overlap and ease interpretation. (B) MIC values for each strain containing the described mutations in *gyrA*. MIC datapoints have been jittered in both plots to prevent overlap and ease interpretation. MIC=minimum inhibitory concentration.

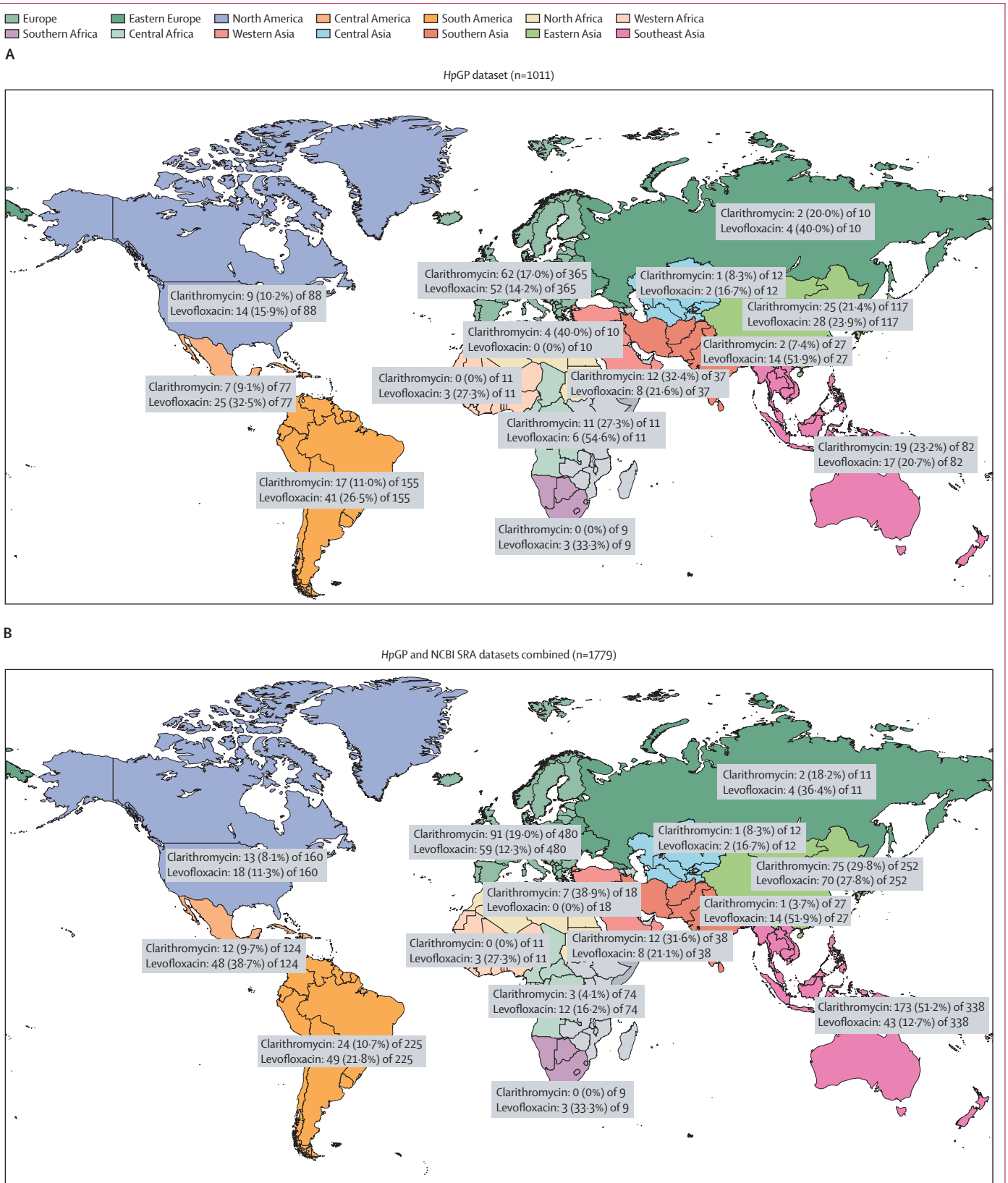


Figure 5: **Global prevalence of clarithromycin and levofloxacin resistance**
(A) Prevalence per geographical region within the *Hp*GP dataset. (B) Prevalence per region combining the *Hp*GP dataset with 768 strains available in the NCBI SRA database. *Hp*GP=*Helicobacter pylori* Genome Project. NCBI SRA=US National Center for Biotechnology Information Sequence Read Archive database.

without known antimicrobial resistance mutations. The resistance prevalence to at least one of the antimicrobials with validated mutations in these regimens ranged from 0·6% (six of 1011) to 21·8% (221 of 1011), whereas bismuth and levofloxacin-based therapy reached 24·5% (248 of 1011).

Discussion

Using a diverse and international collection of *H pylori* strains, we showed that resistance to clarithromycin and levofloxacin can be predicted with very high accuracy from a defined catalogue of genomic mutations. We also provide a highly curated dataset composed of 1011 strain genomes, 419 of which were phenotyped for clarithromycin and levofloxacin susceptibility by the same reference laboratory. Resistance rates to clarithromycin and levofloxacin greater than or equal to 15% are considered high by clinical guidelines.[2] Using the *Hp*GP dataset and other publicly available whole-genome sequences, we showed that resistance patterns predicted from genomes were highly similar to those derived from surveillance based on phenotypic data for the corresponding time period.[17]

A key finding from our study is that a few mutations can explain resistance to clarithromycin and levofloxacin, in contrast with other pathogens, which often involve a high diversity of antimicrobial resistance-associated mutations.[31] These few, strongly explanatory mutations should facilitate the design of molecular diagnostic tests, often limited by the number of mutations they can scan for each targeted gene. Additionally, we provide valuable information about those mutations with more explanatory power or geographical prevalence so they can be prioritised in molecular tests and guide empirical treatments. For clarithromycin, the *23S rRNA* 2143A→G mutation explains 85% of resistant strains in the study dataset. Other *23S rRNA* gene mutations, such as 2182T→C (T2182C), have occasionally been reported in clarithromycin-resistant *H pylori* isolates, but their relevance is still under debate.[32,33] It is possible that previous studies included mixed populations of isolates susceptible and resistant to clarithromycin and harbouring *23S rRNA* mutations that were associated or not with resistance.[34–36] As in Burucoa and colleagues' study,[32] our findings are based on single colonies. The mutation with the highest explanatory power for levofloxacin resistance (*gyrA* N87K) accounted for 35·8% of resistant strains in the study dataset. However, this observation does not mean that unknown genomic regions or mutations cannot become more relevant in the future or that rare mutations might have a role at a local level. Genomic surveillance at different levels will help to complement the information we obtained from the *Hp*GP.

Notably, our study generated MIC data from a centralised reference laboratory. With these granular results, we found that the *23S rRNA* 2142A→G variant is associated with a higher MIC value than 2143A→G. By contrast, *gyrA* variants in D91 have lower MIC values than in N87, and strains with two simultaneous mutations have the highest MICs. Differences in MICs between different mutations can have both clinical and experimental implications, as is being recognised for isoniazid in *M tuberculosis*.[37] Given the unreliable reproducibility of antimicrobial susceptibility testing outside reference laboratories, MICs close to the cutoff used for differentiating wild-type from mutated isolates might lead to wrong categorisation in vitro.

Our analysis by global regions identified geographical differences in resistance prevalence. For example, up to 51·2% of strains were resistant to clarithromycin in southeast Asia, whereas levofloxacin resistance was below 13%. By contrast, more than half of the strains from south Asia were resistant to levofloxacin but fewer than 5% were resistant to clarithromycin. These results are consistent with existing literature. In their meta-analysis, Savoldi and colleagues[17] reported the resistance prevalence to different antibiotics for the different WHO-defined regions. By grouping the data from the present study using the same criteria, we observed similar trends of prevalence (appendix 2 p 20), confirming that molecular markers are, in our dataset, good predictors of AMR. In addition, we provide data from Africa, a region with little information available and where we see relevant differences compared with other countries. For example, no resistance to levofloxacin was observed in north Africa whereas we found a prevalence of more than 30% in southern Africa. According to a 2024 meta-analysis,[38] variations in the prevalence of AMR rates in *H pylori* in different continents, such as those shown in our and other studies, could have arisen from divergent treatment guidelines in these regions. Our data can help to conduct surveillance studies, inform local diagnosis, and design treatment guidelines by region, since in areas where individual susceptibility testing is not available but the prevalence of clarithromycin resistance is high (≥15%) or unknown, the recommended first-line treatment is bismuth-quadruple therapy.[2]

Using the globally representative *Hp*GP dataset, we could predict the putative success of different common eradication regimens. However, our analysis is limited by the absence of mutations to predict resistance to metronidazole and amoxicillin. In fact, our estimated prevalence ranges between 0·6% and 21·9% for these antibiotics, whereas we obtain a prevalence of resistance of 24·5% in the case of the only regimen that does not include either of these drugs (bismuth-based and levofloxacin-based therapy). These results suggest the need to identify clinically relevant mutations for the drugs used in the regimens, as has been done in this work on *23S rRNA* mutations for clarithromycin and *gyrA* mutations for levofloxacin. The validation should include mutations published in the literature as we show that some are highly prevalent in the *Hp*GP dataset and thus unlikely linked to resistance (eg, T31E in *rdxA*; appendix 2 p 11). Our results suggest that several empirical regimens are compromised by different degrees of resistance and showcase the role of genomic-based susceptibility predictions, both for surveillance and for testing. For example, alternative empirical regimens could be being used depending on the prevalence of

resistance.[39] However, they must be guided by local prevalence or resistance estimates; here, we show how genomic surveillance could be a way to inform those regimens. More generally, we think that our data suggest that molecular testing tools, including genomics, can be used to inform susceptibility-guided regimens.

Although the *Hp*GP consortium made a considerable effort to generate a global, representative dataset, gaps in geographical representation remain. Nevertheless, a recent study that followed a similar approach, including the *Hp*GP sequences, reached similar conclusions.[12] In both analyses, locally prevalent or globally rare mutations might still need to be identified. Our analysis should be interpreted as a confirmation of the association with resistance of the mutations present in the *Hp*GP dataset. However, a limitation of this study is that temporal resistance prevalence trends were not considered, which might affect the interpretation of changes over time. Likewise, our estimation of resistance associated with regimens is based on published antimicrobial resistance-conferring mutations, which have not been extensively tested and might lead to overestimation or underestimation of resistance. Thus, we conducted a conservative analysis, in which only mutations that are clearly linked to a resistant phenotype are considered. As such, our estimation of resistance linked to regimens is likely to be a lower bound of the true prevalence. Nonetheless, our study shows that molecular markers are good predictors of phenotypic resistance to clarithromycin and levofloxacin. This is relevant as antimicrobial susceptibility testing is notoriously difficult for *H pylori* and has reproducibility issues, particularly outside reference laboratories.

In summary, we provide a list of validated mutations associated with clarithromycin and levofloxacin resistance in *H pylori* and in some cases to different MICs. Those mutations can be ranked by their predictive value and geographical region to inform the development of global and locally based diagnostics. When extending our genomic predictions of susceptibility to other drugs such as rifabutine or tetracycline, we showed that many patients treated with empirical treatment are likely to harbour strains with resistance to at least one drug. Finally, we provide a publicly available, highly accurate, phenotyped and genotyped global dataset of strains. Future studies should aim to expand available datasets for resistance to other drugs and increase the geographical breadth of the sampling by leveraging novel approaches for genotype–phenotype mapping in drug resistance.

Contributors

FM, MCC, PL, and IC conceptualised the study. MCC, PL, and IC obtained the funding and supervised this analysis. LB, AD, and AG measured the minimum inhibitory concentrations. FJM-M, ÁC-O, and VF conducted bioinformatics and statistical analyses of sequenced and phenotyped data. FJM-M, ÁC-O, VF, IC, MCC, MJ, and PL were responsible for the validation of the results. MCC, PL, and IC have directly accessed and verified the data. FJM-M, ÁC-O, IC, MCC, and PL were responsible for writing the manuscript. All *Hp*GP Research Network authors critically revised and approved the final version of the manuscript, had full access to all the data in the study, and accepted responsibility for submitting it for publication.

Declaration of interests

We declare no competing interests.

Data sharing

*Hp*GP genome sequences are publicly available at the National Center for Biotechnology Information under project PRJNA529500. All scripts used in the study are publicly available at https://gitlab.com/tbgenomicsunit/hpgp_antimicrobialresistance.

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