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Comparison of Culture With Antibiogram to Next-Generation Sequencing Using Bacterial Isolates and Formalin-Fixed, Paraffin-Embedded Gastric Biopsies

Kristina G. Hulten[1], **Robert M. Genta**[2,3], **Ira N. Kalfus**[4], **Yi Zhou**[5], **Hongjun Zhang**[5], **David Y. Graham**[6]

[1]Department of Pediatrics, Baylor College of Medicine, Houston, Texas

[2]Inform Diagnostics, Irving, Texas

[3]Department of Pathology, Baylor College of Medicine, Houston, Texas

[4]M2G Consulting, New York, New York

[5]American Molecular Laboratories, Vernon Hills, Illinois

[6]Department of Medicine, Michael E. DeBakey VA Medical Center and Baylor College of Medicine, Houston, Texas

Abstract

BACKGROUND & AIMS: The decline in *Helicobacter pylori* cure rates emphasizes the need for readily available methods to determine antimicrobial susceptibility. Our aim was to compare targeted next-generation sequencing (NGS) and culture-based *H pylori* susceptibility testing using clinical isolates and paired formalin-fixed, paraffin-embedded (FFPE) gastric biopsies.

Correspondence Address correspondence to: David Y. Graham, MD, Department of Medicine, Michael E. DeBakey Veterans Affairs Medical Center, RM 3A-318B (111D), 2002 Holcombe Boulevard, Houston, Texas 77030. dgraham@bcm.edu.

Conflicts of interest

The authors disclose the following: Kristina G. Hulten received a grant from RedHill for performing cultures and antimicrobial susceptibilities. Robert M. Genta has performed the histopathology studies related to this project and has received consulting fees from RedHill. David Y. Graham is a consultant for RedHill Biopharma and Phathom Pharmaceuticals regarding novel *Helicobacter pylori* therapies. Ira N. Kalfus was the medical director at RedHill Biopharma responsible for the conduct of the study. Yi Zhou and Hongjun Zhang are employees of American Molecular Laboratories, which developed the PyloriAR NGS system. This study was performed using material from a clinical trial by RedHill BioPharma.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2021.07.012.

CRediT Authorship Contributions

David Y. Graham, MD (Conceptualization: Equal; Data curation: Equal; Formal analysis: Equal; Funding acquisition: Equal; Investigation: Equal; Methodology: Equal; Project administration: Equal; Supervision: Lead; Writing – original draft: Equal; Writing – review & editing: Equal). Kristina G. Hulten, PhD (Conceptualization: Equal; Data curation: Lead; Formal analysis: Equal; Investigation: Equal; Methodology: Equal; Project administration: Equal; Resources: Equal; Supervision: Lead; Writing – original draft: Equal; Writing – review & editing: Equal). Robert M. Genta, MD (Data curation: Equal; Formal analysis: Equal; Investigation: Lead; Resources: Equal; Supervision: Equal; Writing – original draft: Equal; Writing – review & editing: Equal). Ira N. Kalfus, MD (Conceptualization: Equal; Formal analysis: Equal; Funding acquisition: Equal; Investigation: Equal; Resources: Equal; Writing – original draft: Equal; Writing – review & editing: Equal). Yi Zhou, PhD (Data curation: Equal; Formal analysis: Equal; Investigation: Equal; Methodology: Equal; Resources: Equal; Software: Equal; Validation: Equal; Writing – original draft: Equal; Writing – review & editing: Equal). Hongjun Zhang, MD, PhD (Conceptualization: Equal; Data curation: Equal; Formal analysis: Lead; Funding acquisition: Equal; Investigation: Equal; Methodology: Lead; Project administration: Equal; Resources: Equal; Software: Lead; Supervision: Equal; Validation: Lead; Writing – original draft: Equal; Writing – review & editing: Equal).

METHODS: *H pylori* isolates and FFPE tissues were tested for susceptibility to amoxicillin, clarithromycin, metronidazole, levofloxacin, tetracycline, and rifabutin using agar dilution and NGS targeted to 23S *rRNA*, *gyrA*, *16S rRNA*, *pbp1*, *rpoB* and *rdxA*. Agreement was quantified using *k* statistics.

RESULTS: Paired comparisons included 170 isolates and FFPE tissue for amoxicillin, clarithromycin, metronidazole, and rifabutin and 57 isolates and FFPE tissue for levofloxacin and tetracycline. Agreement between agar dilution and NGS from culture isolates was very good for clarithromycin ($k = 0.90012$), good for levofloxacin ($k = 0.78161$) and fair for metronidazole ($k = 0.55880$), and amoxicillin ($k = 0.21400$). Only 1 isolate was resistant to tetracycline (culture) and 1 to rifabutin (NGS). Comparison of NGS from tissue blocks and agar dilution from isolates from the same stomachs demonstrated good accuracy to predict resistance for clarithromycin (94.1%), amoxicillin (95.9%), metronidazole (77%), levofloxacin (87.7%), and tetracycline (98.2%). Lack of resistance precluded comparisons for tetracycline and rifabutin.

CONCLUSIONS: Compared with agar dilution, NGS reliably determined resistance to clarithromycin, levofloxacin, rifabutin, and tetracycline from clinical isolates and formalin-fixed gastric tissue. Consistency was fair for metronidazole and amoxicillin. Culture-based testing can predict treatment outcomes with clarithromycin and levofloxacin. Studies are needed to compare the relative ability of both methods to predict treatment outcomes for other antibiotics.

Keywords

Helicobacter pylori; Next-Generation Sequencing; Culture; Bacterial Resistance; Formalin-Fixed Tissue

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Infectious disease therapies have traditionally been susceptibility-based, with treatment outcomes being defined by cure rates. *Helicobacter pylori* is an outlier, as most often therapy is prescribed without reference to local susceptibility data and the results are often suboptimal.[1,2] Both infectious disease and gastroenterology describe therapy as “empirical.” The formal definition of the word *empirical* is “to be based on, concerned with, or verifiable by observation or experience rather than theory or pure logic.”[3] In infectious disease therapy, empiric therapy is used when the prevalence of susceptible organisms in the population is known to be high and high cure rates can be expected, or when therapy is given before the results of culture become available, with the understanding that the therapy will be revised (eg, to a more narrow-spectrum antibiotic) when the microbiology results become available.[4] In *H pylori* therapy, empiric has a different connotation, as it is often used as a synonym for *hope*, defined as “a feeling of expectation and desire for a certain thing to happen,” and should probably be called “hopeful” (feeling or inspiring optimism about a future event) rather than empiric therapy.

The general unavailability of culture-based susceptibility testing for *H pylori* has resulted in the almost universal reliance on hopeful (empiric) therapy and a high proportion of treatment failures.[2,5] It has become clear that it is not possible to reliably cure *H pylori* infections without relying on direct or indirect susceptibility (ie, outcomes) data.[1] Unsatisfactory treatment outcomes coupled with increasing worldwide antimicrobial resistance have resulted in a movement toward the adoption of the general principles of

antimicrobial therapy[4,6] and of antimicrobial stewardship, which is based on the use of optimized susceptibility-based therapies designed to reliably achieve high cure rates while simultaneously minimizing the development of resistance.[7,8]

Traditional culture-based susceptibility testing requires access to gastric contents and although minimally invasive approaches for collection of gastric juice, mucus, or tissue using tubes, strings, brushes, or forceps have been developed, testing is rarely done. Successful culture-based testing also requires appropriate biopsy handling, special transport conditions, and an experienced laboratory, and, even when this system works effectively, results are rarely available within 2 weeks. Molecular-based testing is being used increasingly to confirm the presence of an *H pylori* infection, as well as to identify alterations in the genetic sequence of microbial genes resulting in antimicrobial resistance.[9–11] Modern molecular testing is rapid, with a turnaround time of less than 1 week and can provide susceptibility results for multiple antibiotics.[12,13] Testing can use gastric contents (ie, gastric juice, mucus, or mucosal biopsies), formalin-fixed, paraffin-embedded (FFPE) tissue, feces, or repurposing of tissue taken for histology or rapid urease tests.

Until recently, the majority of molecular testing was designed to identify single or small sets of mutations in specific genes or in limited regions to detect resistance to clarithromycin or fluoroquinolones, either singly or simultaneously.[14,15] However, *H pylori* has enormous genetic diversity, with high rates of mutation and recombination events, such that the mechanisms of antimicrobial resistance are both heterogeneous and intricate. For example, mutations or DNA sequence changes associated with metronidazole resistances have been described in multiple genes and regions, including the *rdxA*, *frxA*, *fdxB*, and *recA* genes, as well as nucleotide alterations within genes or regions, such as nonsense, missense, frameshift mutations, deletions, and insertions.[16–18] Because of this complexity, polymerase chain reaction (PCR)-based tests using hybridization or enzymatic analytic methods are often not feasible for simultaneously testing of multiple antibiotics or for reliably detecting resistance to metronidazole. Sanger sequencing provides a relatively accessible approach and has been used for resistance analysis, but the method is limited by low throughput and lacks the sensitivity to detect mutations/variances with frequencies <10%–20%.[19]

Most commercially available kits for molecular testing for *H pylori* resistance are designed for use with culture isolates or fresh samples. In contrast, next-generation sequencing (NGS) technology can be used for the detection of mutation/DNA variances in a wide variety of indications, such as cancer precision medicine, genetic tests, and infectious diseases. Molecular testing has become increasing available and affordable, with its use being enhanced by technical developments regarding error correction strategies in NGS to allow accurate identification of low-level genetic variations.[20] For example, NGS is now used successfully in drug susceptibility profiling for *Mycobacterium tuberculosis*.[21]

This study compared an NGS method (PyloriAR NGS, developed by American Molecular Laboratories, Vernon Hills, IL) for the detection of antimicrobial resistance markers from 2 different sources of *H pylori: H pylori* isolates cultured from gastric biopsies and DNA extracted from FFPE tissue blocks containing gastric mucosal biopsies. The molecular results were compared with a gold standard—susceptibility testing by agar dilution—and

included the following 6 antimicrobial drugs: amoxicillin, clarithromycin, levofloxacin, metronidazole, tetracycline, and rifabutin.

Methods

The goal of this study was to compare susceptibility results obtained by culture and agar dilution with molecular-based analysis of *H pylori* antimicrobial resistance markers using isolates and tissue blocks of gastric mucosal biopsies. Gastric biopsy specimens were obtained from the antrum and corpus, respectively, from treatment-naïve adults (aged 18–70 years) who participated in a nationwide US *H pylori* eradication study clinical trial.[22] In all subjects, the presence of *H pylori* infection was originally determined by ^{13}C urea breath tests, and confirmed by upper endoscopy with positive culture, histology, or rapid urease or *Campylobacter*-like organism test.

Study Design

Cases with both positive cultures and matching paraffin-embedded gastric biopsies were included. Before sending, *H pylori* isolates were re-randomized at Texas Children's Hospital. Paraffin-embedded gastric mucosal biopsies were coded separately at the Miraca Life Sciences Laboratory. Both sets were sent for molecular testing and after culture and molecular assays, the data sets were unlocked and the results were compared. The following issues were addressed: how well the NGS predicted susceptibility corresponded to the culture-based susceptibility results from the same isolates; and how well NGS predicted susceptibility obtained from formalin-fixed gastric tissue corresponded to *H pylori* culture–based susceptibility data obtained from the same individuals. As antral and corpus samples were combined, all results are reported per patient not in relation to individual biopsy sites.

Samples

Antimicrobial susceptibility testing.—Minimal inhibitory concentrations (MICs) for amoxicillin, clarithromycin, metronidazole, and rifabutin were obtained as part of the treatment study. Because the original clinical study did not include susceptibility testing for tetracycline or levofloxacin, available isolates that could be revived from frozen stocks and that grew in sufficient density for agar dilution testing were tested and included in this study. Clinical and Laboratory Standards Institute methods and guidelines were followed.[23,24] The detailed methods were described previously.[22,25]

Antibiotics were obtained from US Pharmacopeia, North Bethesda, MD. *H pylori* ATCC 43504 was included as a control. Clinical and Laboratory Standards Institute and the European Committee on Antimicrobial Susceptibility Testing (www.eucast.org) interpretive break points were used to determine resistance rates.

Gastric Biopsies

Sets of biopsies (1 each from antrum and corpus) were obtained for rapid urease or Campylobacter-like organism test, histopathology (Miraca Life Sciences, Irving, TX [now Informed Diagnostics]) and culture and susceptibilities (Texas Children's Hospital, Houston,

TX) were published as part of a clinical trial comparing rifabutin triple therapy with proton pump inhibitor (PPI)-amoxicillin dual therapy.[22]

Next-Generation Sequencing–Based Molecular Methods for Helicobacter pylori Antibiotic Resistance Analysis

NGS was performed (PyloriAR; American Molecular Laboratories, Chicago, IL) to identify mutations or variances of the *H pylori* DNA present in fixed tissue and culture isolates. Markers of antibiotic resistance associated with 6 *H pylori* genes—23S *rRNA*, *gyrA*, *16S rRNA*, *pbp1*, *rpoB* and *rdxA*—were used (Table 1).[16,26–31]

The PyloriAR NGS method uses proprietary designs to enrich the targeted areas to ensure the coverage. This is especially important in FFPE samples that contain fragmented DNA. The NGS assay was validated and the major parameters were set as follows: correct identification of mutations or variances required a minimum of 500 properly mapped reads and a minimum of 25 variant reads. PylorAR NGS protocols including the following steps: DNA extraction, real-time PCR for *H pylori* confirmation and quantitation, NGS library preparation, sequencing, and data analysis, which are detailed below.

Sample Processing and DNA Extraction

DNA preparation of tissue samples.—Five 10-μm sections were cut from each FFPE tissue block (which contained both the antral and corpus biopsy) on a microtome. The DNA was extracted from the sections using a Qiagen QIAcube processor and FFPE tissue kits with a modified protocol. Briefly, tissue sections were deparaffinized and placed in a tube with lysis buffer. Proteinase K was added and they were incubated at 56°C for 60 minutes, followed by 90°C for an additional 60 minutes. The lysed samples were then mixed with buffer and DNA was extracted using an automated Qiagen QIAcube system. To evaluate the quality of the extracted DNA from FFPE tissue, a multiplex PCR qualification assay with the amplification of the human GAPDH gene encoding glyceraldehyde-3-phosphate dehydrogenase was developed and used. The multiplex PCR has a set of PCR primers capable of producing an amplicon ladder of 100, 200, 300, 400, and 500 base-pair fragments. The DNA recovered from FFPE gastric biopsy samples was considered good if the test produces 2–5 bands of the amplicon ladder and was acceptable for the NGS analysis.

DNA preparation of *H pylori* isolates.—Bacterial pellets were harvested from fresh cultures of the combined antral and corpus biopsies on agar plates and resuspended in 100 μL sterile 0.9% NaCl in the Infectious Disease Research Laboratory at Texas Children's Hospital. The culture samples were relabeled and sent in a blinded fashion to the molecular laboratory for NGS analysis. Frozen subcultures were sent to the molecular diagnostic laboratory on dry ice. For DNA extraction 75 μL of each sample was mixed with lysis buffer and proteinase K, incubated at 56°C for 30 minutes followed by incubation at 90°C for 5 minutes. The lysed samples were then loaded on the Qiagen QIACube for DNA extraction. The DNA extracted from tissue blocks and culture isolates was quantified with Qubit 2.0 (Thermo Fisher Scientific) and stored at 4°C temporarily for immediate downstream testing or stored at −20°C for analysis at a later time.

Real-Time Quantitative Polymerase Chain Reaction for H pylori *Confirmation and Quantitation*

To confirm the presence of and quantitate *H pylori* DNA from the tissue blocks and culture isolates, a multiplex real-time PCR was performed targeting the *H pylori* 23S rRNA gene. Human GAPDH gene was used as internal control for the tissue samples. PCR was carried out on QuantStudio 3 (Applied Biosystems) with the initial denaturation at 95°C for 3 minutes, followed by 40 cycles consisting of denaturation at 95°C for 3 seconds and annealing/extension at 60°C for 30 seconds. Data analysis was performed with the QuantStudio Design & Analysis Software, version 1.4.3, installed as part of the Applied Biosystems device. Eligible samples with sufficient DNA were moved to the next step for NGS library preparation.

NGS library preparation.—The method uses a targeted and paired-end sequencing library preparation method. A multiplex PCR was then used to amplify the targeted areas from 6 genes of the *H pylori* genome, including 23S rRNA, *gyrA*, *rdxA*, *pbp1*, 16S rRNA, and *rpoB*, see Table 1. The obtained amplicons from each amplification reaction were purified with the Agencourt AMPure XP PCR Purification system following the manufacturer's instructions. The adapters and indexes were added and then followed by multiple clean-up steps. The library prepared for *H pylori* NGS is subject to quality control with fragment analysis, which ensures the appropriate DNA fragments are generated. A representative set of library samples are run on Bioanalyzer 2100 (Agilent). For the *H pylori* library, the peak approximately 300 base pair is expected.

Sequencing and Data Analysis

The libraries were pooled and loaded on the MiSeq platform (Illumina, Inc) for sequencing. The FASTQ formatted sequence data were generated with the MiSeq Reporter Analysis Software after the sequencing run. The Q-score ≥30 of sequenced reads ranging from 85% to 93% was used to confirm the high quality of the sequencing runs and its acceptability for further analysis. The commercial software NextGENe (SoftGenetics) was used to analyze the sequence data to identify antibiotic resistance mutations. The FASTQ data were initially converted to a standard fasta format and low-quality reads were trimmed or removed. The remaining clean reads were aligned and mapped to corresponding genes from the reference *H pylori* 26695 genome sequence (GenBank accession NC_000915). In order to identify mutations or variances correctly and minimize sequencing errors, a minimum of 500 properly mapped reads were required for the detection of variance at a 5% frequency. NGS can also potentially identify mixed infections using the ratio of wild-type and resistant strains in the testing samples based on the variant frequency (see Supplementary Material). In this study, the samples were reported and counted as resistant when 5% or more mutations or variances associated with resistance were detected.

Ethical Considerations

The original study from which the samples were obtained was conducted in accordance with ethical principles of the Declaration of Helsinki and applicable regulatory requirements. The study protocol and all amendments were approved by the respective Institutional Review

Boards of participating institutions. All patients provided written informed consent before participating in the study.[22] This molecular comparison study did not include patient data and only used deidentified tissue and bacterial isolate preparations, and was approved by the Institutional Review Board for Baylor College of Medicine and affiliated hospitals. All of the authors have had full access to all of the data.

Statistical Analyses

The comparisons between the results of the 2 of methods (molecular detection of resistance markers vs agar dilution [gold standard]) was based on the established breakpoints, which is referred to here as the MIC. The comparisons between the results of the 2 methods to predict reference/susceptibility were done using the statistical program at SciStat. Agreement was quantified by the k statistic, where $k = 1$ when there is perfect agreement between the classification systems; $k = 0$ when there is no agreement better than chance; and k is negative when agreement is worse than chance. The k values with standard error and 95% confidence interval are provided.

Results

Cases in the Study

Because of the double-blinded design of this study and the availability of tissue blocks and culture isolates, the number of isolates and tissue blocks for which paired comparisons could be done was less than the total number included in the clinical study. The presence of *H pylori* DNA was confirmed in all tissue blocks and isolates received by the molecular diagnostic laboratory using real-time PCR, and they were then processed further for NGS analysis. A total of 170 tissue blocks had paired isolates and corresponding MIC results for clarithromycin, metronidazole, amoxicillin, and rifabutin; and a subset of 57 tissue blocks had paired isolates and corresponding MIC results for fluoroquinolones and tetracycline. The sequences of antibiotic resistance-related genes or regions were retrieved from the annotated genomes to identify potential antibiotic-resistance mutations. Mutations in genes previously reported to be associated with clarithromycin, levofloxacin, metronidazole, amoxicillin, tetracycline, and rifampin resistance were examined and the correlation with the phenotypic results was determined. In all instances, at least 500 reads were reached by NGS for the gene regions and hot spot where the variants were called.

Comparison of the Results of Culture Susceptibility (Minimal Inhibitory Concentration) and Resistance Marker Testing by Next-Generation Sequencing on Culture Isolates and Formalin-Fixed, Paraffin-Embedded Tissues

Clarithromycin.—Clarithromycin resistance of *H pylori* has been related to *23S* rRNA gene mutations. The most common mutation (approximately 90%) in clarithromycin-resistant strains is an adenine to guanine transition at position 2142 and 2143 and an adenine to cytosine transversion at position 2142.[28] There are 2 copies of 23S rRNA genes in the *H pylori* genome, but it was considered as resistant if the mutations were present in at least 1 copy. Comparison of the determination of resistance using MIC and PyloriAR NGS testing of frozen *H pylori* isolates (n = 170) had strong agreement ($k = 0.90012$) with a significant correlation coefficient ($P < .0001$) (Table 2). The results comparing MIC and PyloriAR NGS

molecular methods to test FFPE tissue (n = 170) also had a good agreement (k = 0.81236) (P < .0001) (Table 3).

PyloriAR NGS results predicted *H pylori* resistance in isolates (susceptible or resistant) with a 97.1% accuracy compared to the gold standard (agar dilution) with a sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of 93.3%, 97.9%, 90.32%, and 98.6%, respectively. From the FFPE tissues, PyloriAR NGS predicted *H pylori* resistance in 94.1% of samples compared to the gold standard (MIC) with a sensitivity, specificity, PPV, and NPV of 93.3%, 94.3%, 77.9%, and 98.5%, respectively.

Amoxicillin.—Amoxicillin resistance is still rare in *H pylori* but, when present, has primarily been linked to mutations in the gene *pbp1* with a mutation at amino acid Ser414Arg/Asn. PyloriAR NGS applied Ser414Arg/Asn as the marker to predict resistance. Based on the k statistic, the agreement between the isolates or FFPE tissue compared to the gold standard was fair but significant (both k = 0.214) (P < .05). The accuracy of PyloriAR NGS in predicting *H pylori* resistance from culture isolates and FFPE tissue was the same: 95.9% (Tables 2 and 3). In addition to Ser414Arg/Asn mutation in the gene *pbp1*, other mutations, as shown in Supplemental Table 1, were observed in isolates and/or tissues; none were specifically associated with resistance. In 41 of the 170 tissue samples, D479E, T593A/K, and G595S were present; more often in susceptible than in resistant samples. In 170 isolates, D479E, T593A/K, and G595S mutations were present in 53 samples; more often in susceptible than in resistant samples (Supplementary Tables 2 and 3). Overall, compared to the gold standard, PyloriAR NGS was unable to confirm resistance in 7 of 170 isolates and paired 170 tissue samples. However, it is important to note and, as elaborated in the Discussion, in the clinical study from which the samples were obtained the agar dilution results often failed to reliably predict outcomes with the dual PPI-amoxicillin therapy.[22]

Metronidazole.—Any mutation, insertion/deletion in gene *rdxA* resulting in a stop codon and amino acid frameshift can result in metronidazole resistance.[32] The mutation/DNA variances that have been associated with resistance include R16H, M21R/T/V, C19Y, P51L, A67V, C87Y, C140Y, C184Y, and G163D/S/V.[29,33,34] The resistance results comparing agar dilution and PyloriAR NGS molecular testing with frozen H pylori isolates (n = 170) and the resistance results between agar dilution and PyloriAR NGS molecular testing with FFPE tissue (n = 170) had moderate agreement (k = 0.55880 and k = 0.54645, respectively) (P < .0001) (Tables 2 and 3). The predicted accuracy of PyloriAR NGS was 77.6% and 77% from culture isolates and FFPE tissues, respectively.

Rifabutin.—Resistance of *H pylori* to rifabutin results from mutations in the rpoB gene.[35] There were no rifabutin-resistant cases among the 170 tissue samples by culture MIC and PyloriAR NGS. One sample was resistant by PyloriAR NGS molecular testing among the isolates, but was judged susceptible in the paired FFPE sample on the same subject. Based on these data, PyloriAR NGS molecular testing for predicting *H pylori* susceptibility to rifabutin achieved the specificity of 99.4% and 100% in culture isolates and FFPE tissues respectively (Tables 2 and 3). However, the lack of rifabutin resistance among the samples tested limits this analysis.

Levofloxacin.—Resistance to levofloxacin results from mutations in the *gyrA* gene, most commonly caused by a point mutation at amino acid 87 (Asn to Lys or Ile) or 91 (Asp to Gly, Asn or Tyr).[35] There were 57 cases with FFPE tissue blocks and paired culture isolates with corresponding agar dilution results. There was a good agreement between agar dilution results and PyloriAR NGS of isolates, and between agar dilution results and PyloriAR NGS of FFPE tissues ($k = 0.78163$ and $k = 0.74953$, respectively) ($P < .0001$) (Tables 2 and 3).

Compared to the gold standard, PyloriAR NGS of isolates identified *H pylori* resistance with an accuracy of 89.5% with sensitivity, specificity, PPV, and NPV of 93.9%, 83.3%, 88.6%, and 90.9%, respectively. PyloriAR NGS of FFPE tissue identified *H pylori* resistance with an accuracy of 87.7% with sensitivity, specificity PPV, and NPV of 87%, 87.5%, 90.6% and 84%, respectively.

Tetracycline.—Tetracycline resistance has been associated with 3 nucleotide changes at position 926–928 in *16S rRNA* gene of *H pylori*. If there is only 1 nucleotide change, the resistance level is low, whereas the triple mutation, such as AGA926–928TTC, results in high-level tetracycline resistance.[30,36] NGS required at least 2 mutations in the *16S RNA* gene to predict resistance to tetracycline. Because there are 2 copies of *16S rRNA* genes in the *H pylori* genome, a sample was considered resistant if the mutations were present in either or both copies. PyloriAR NGS identified tetracycline resistance in in none of the 57 culture isolates and 1 of 57 (2.8%) isolates were resistant by agar dilution. Based on the data, the PyloriAR NGS predicted *H pylori* resistance to tetracycline with an accuracy of 98.2% in both isolates and FFPE tissues (Tables 2 and 3).

Discussion

With the exception of *H pylori*, antimicrobial therapy for infectious diseases is either directly or indirectly susceptibility-based, consistent with increasing pressure to uniformly use the principles of antimicrobial stewardship for managing infectious disease therapy.[8] The movement toward tailored or susceptibility-based *H pylori* therapies is also reflected in the more than 70 citations in PubMed,[37–39] as well as editorials on this topic.[40] In China, where *H pylori* antimicrobial resistance is especially prevalent, a number of studies have confirmed that using therapies based on an individual's susceptibility test results provides highly effective outcomes, despite a high local prevalence of antimicrobial resistance.[41–44]

The generally poor cure rates obtained with empiric use of the currently most popular triple therapies for *H pylori* infection reflect both the global increase in prevalence of antimicrobial resistance and the lack of availability of culture-based susceptibility testing.[1,2] Molecular-based tests designed to identify antimicrobial resistance are now commercially available and theoretically could provide up-to-date local and regional *H pylori* susceptibility data to enhance cure rates and limit the development of antimicrobial resistance.[4,7,8,45] Available PCR-based tests are usually designed to detect 1 or a few individual targets.[11] In contrast, NGS methods can simultaneously identify DNA mutations or variances of *H pylori* genes responsible for resistance in all of the commonly used anti–*H pylori* antimicrobials.[13,46]

We used the PyloriAR NGS test (American Molecular Laboratories), which can simultaneously provide information regarding resistance in 6 commonly used anti–*H pylori* antimicrobials (Table 1). This method has advantages over whole genome sequencing, which, although it is an excellent research tool to identify new mutations, especially when using bacterial colonies, it is currently neither practical nor cost-effective for routine clinical use.[47,48] The presence of fragmented DNA also makes whole genome sequencing more difficult when applied to FFPE tissue. The targeted sequencing approach using NGS is currently a more efficient and cost-effective approach compared to whole genome sequencing.

PyloriAR NGS uses proprietary designs designed to enrich and cover multiple genes of interest and large regions that DNA mutation/variance that can cause resistance. This approach ensures sufficient sequencing coverage of FFPE tissues containing fragmented DNA or crosslinks between DNA and protein or other macromolecules. The advantages of this technology include the wide range of sample types that can be used, a high rate of successful sequencing (100% in this study) and rapid availability of results. Furthermore, for mixed samples containing both susceptible and resistant strains, the method can detect and predict the frequency of a mutation within the sample (see Supplementary Material, Supplementary Table 4). However, limitations do exist, including that it focuses on already known genomic regions for antimicrobial resistance, as the predictive value might be somewhat reduced because of DNA fragmentation of FFPE tissue samples.

This study compared the results of culture-based and molecular-based susceptibility testing of 6 antibiotics using both FFPE biopsies and *H pylori* isolates obtained from the same subjects. Overall, molecular-based testing agreed with the MIC results obtained by agar dilution for clarithromycin and levofloxacin. The comparison between MIC and molecular results was less consistent for metronidazole, and the correlation between culture-based susceptibility testing and molecular testing with amoxicillin was relatively poor. Tetracycline and rifabutin resistance was rare to absent in our sample, which limited the evaluation. However, for susceptible isolates, both culture and molecular testing were highly concordant.

Clarithromycin, levofloxacin, and, presumably, rifabutin resistance involves single targets that govern susceptibility vs resistance; clinically, resistance eliminates the antibiotic's beneficial effects and reduces a triple therapy (eg, a PPI, clarithromycin, and amoxicillin) to PPI-amoxicillin dual therapy. Metronidazole is a prodrug that is activated intracellularly into a toxic version that destroys the bacterial DNA. Metronidazole susceptibility predicts treatment success, but the effect of resistance is not all or none, but depends on the composition of the therapy as well as the metronidazole dosage and duration of therapy. Amoxicillin resistance has been related to alterations in penicillin-binding proteins[49,50] and is still rare among *H pylori* isolates from most regions. To date, no *H pylori* strains have been reported that produce β-lactamases. Our finding that culture-based amoxicillin susceptibility testing and molecular testing correlated poorly was also observed by of Azzaya et al.[51] The relative poor results obtained for amoxicillin and metronidazole are most likely the consequence of an insufficient molecular characterization of these antibiotic resistances. It is possible that genetic alterations in other genes could be associated with

the increased MIC observed for these isolates. Our samples were derived from a study that included a PPI-amoxicillin dual therapy that potentially allowed the effect of amoxicillin resistance to be judged without the presence of a second antibiotic. Importantly, in that PPI-amoxicillin dual therapy study, MIC values for amoxicillin resistance did not reliably predict treatment failure or success.[22] However, outcomes assessment was complicated because the overall cure rate was low (57.7%; 95% confidence interval, 51.2%–64.0%), which was typical for studies of PPI-amoxicillin dual therapy in the United States.[52–55] A pooled analysis of 5 randomized trials of amoxicillin-containing triple therapy showed that phenotypic amoxicillin resistance was associated with a decrease in cure rate.[56]

The majority of the published literature on molecular testing in *H pylori* has been concerned with detection of genotypic resistance unencumbered by comparisons with culture-based results or with clinical outcomes.[57] Ultimately, the comparisons should include the response to treatment, which it often complicated by the use of multidrug regimens. Further studies are needed to identify which genes, or patterns of gene involvement, best correlate with outcome.

In summary, the results of this study suggest that molecular methods can potentially augment or even replace the current in vitro methods for susceptibility testing, which are cumbersome, technically challenging, and time-consuming. As with other genomic-based methods, phenotypic, culture-based susceptibility testing and genomic prediction of resistance do not always correspond. Treatment failure is currently predicted by determining a breakpoint MIC value based on the results of clinical treatment data. Reliable predictive data for *H pylori* therapy are available for only clarithromycin and levofloxacin. Studies in populations with high prevalence of tetracycline, rifabutin, or amoxicillin resistance are needed to help clarify how to best use molecular testing results to reliable predict outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper:

FFPE	formalin-fixed, paraffin-embedded
MIC	minimal inhibitory concentration
NGS	next-generation sequencing

NPV	negative predictive value
PCR	polymerase chain reaction
PPI	proton pump inhibitor
PPV	positive predictive value

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WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Cure rates using empiric therapy for *Helicobacter pylori* infections have declined steadily, stimulating a need for a widely available method for susceptibility testing. Next-generation sequencing (NGS) is a candidate to replace or supplement culture, as it can use isolates, biopsies, formalin-fixed biopsies, or stools to rapidly and reliably identify alterations in the genetic sequence of microbial genes resulting in antimicrobial resistance.

NEW FINDINGS

Compared to agar dilution, NGS reliably determined resistance to clarithromycin, levofloxacin, rifabutin, and tetracycline from clinical isolates and formalin-fixed gastric tissue. Consistency was fair for metronidazole and amoxicillin.

LIMITATIONS

Limitations include lack of sufficient isolates resistant to rifabutin and tetracycline and inability to correlate the susceptibility profiles to the outcomes of therapy.

IMPACT

Molecular-based susceptibility testing has the potential to replace culture-based testing.

Table 1.

Targeted Genes and Mutations Reported to Predict Antibiotic Resistance

Antibiotics	Genes	Mutations and positions
Clarithromycin	23S rRNA	A2142G, A2143G, A2142C
Fluoroquinolones	*gyrA*	A260T (Asn87Ile), T261A, T261G (Asn87Lys), G271A (Asp91Asn), G271T (Asp91Gly)
Metronidazole	*rdxA*	Insertion/deletion causing new stop codon or frame shift Missense mutations: R16HC, M21R/T/V, C19Y, P51L, A67V, C87Y, C140Y, C184Y, G163D/S/V
Amoxicillin	*pbp1*	Ser414Arg/Asn
Tetracycline	16S rRNA	TCT926–928AAG, T926A/C927A, T926A/T928G, T926C/C927A, T926C/T928G and C927A/ T928G
Rifabutin	*rpoB*	Mutation in codon 525 to 545

Table 2.

The Accuracy of PyloriAR Next-Generation Sequencing in Predicating *Helicoobacter pylori* Antibiotic Resistance in Culture Isolates

		Culture (MIC)				Predicative value, %			
Antibiotics	**PyloriAR NGS culture isolate**	**(+)**	**(−)**	**Sensitivity, %**	**Specificity, %**	**PPV**	**NPV**	**Accuracy, %**	**Agreement, *k* coefficient**
Clarithromycin	(+)	28	3						
n = 170	(−)	2	137	93.3	97.9	90.3	98.6	97.1	0.90012 ($P < .0001$)
95% CI	—	—	—	77.9–99.2	93.9–99. 6	75.2–96.6	94.7–99.6	93.3–99	0.8671–0.9253
Amoxicillin	(+)	1	0						
n = 170	(−)	7	162	12.5	100.0	100.0	95.9	95.9	0.21400 ($P = .0051$)
95% CI	—	—	—	0.3–52.6	97.7–100.0		94.7–96.8	91.7–98.3	0.0656–0.3531
Metronidazole	(+)	68	9						
n = 170	(−)	29	64	70.1	87.7	88.3	68.8	77.6%	0.5588 ($P < .0001$)
95% CI	—	—	—	50–79	77.9–94.2	80.2–93.4	61.7–75.2	70.63–83.7	0.4458–0.6543
Rifabutin	(+)	0	1						
n = 170	(−)	0	169	—	99.4	0.00	100.0		—
95% CI	—	—	—	—	96.8–100	—	—		—
Levofloxacin	(+)	31	4						
n = 57	(−)	2	20	93.9	83.3	88.6	90.9	89.5	0.78161 ($P < 0.0001$)
95% CI	—	—	—	79.8–99.3	62.6–95.3	75.9–95	72.1–97.5	78.5–96	0.61689–0.94633
Tetracycline	(+)	0	0						
n = 57	(−)	1	56	0.00	100.0	—	98.2	98.2	—
95% CI	—	—	—	0.00–97.5	93.6–100.0	—	98.2–98.2	90.6–100	—

(+), resistant; (−), sensitive; CI, confidence interval.

Table 3.

The Accuracy of PyloriAR Next-Generation Sequencing in Determining the Antibiotic Resistance of *Helicobacter pylori* in Formalin-Fixed, Paraffin-Embedded Tissues

		Culture (MIC)				Predictive value, %			
Antibiotics	**PyloriAR NGS, FFPE results**	**(+)**	**(−)**	**Sensitivity, %**	**Specificity, %**	**PPV**	**NPV**	**Accuracy, %**	**Agreement, *k* coefficient**
Clarithromycin	(+)	28	8						
Total n = 170	(−)	2	132	93.3	94.3	77.8	98.5	94.1	0.81236 ($P < .0001$)
95% CI	—	—	—	77.9–99.2	89–97.5	63.9–87.3	94.5–99.6	89.4–97.1	0.70076 to 0.92396
Amoxicillin	(+)	1	0						
Total n = 170	(−)	7	162	12.5	100	100	95.9	95.9	0.21400 ($P = .0051$)
95% CI	—	—	—	0.3–52.6	97.7–100.0	—	94.7–96.8	91.7–98.3	−0.13849 to 0.56649
Metronidazole	(+)	68	10						
Total n = 170	(−)	29	63	70.1	86.3	87.2	68.5	77.1	0.54645 ($P < .0001$)
95% CI	—	—	—	60–79	76.2–93.2	79–92.5	61.2–74.9	70.0–83.1	0.42443 to 0.66847
Rifabutin	(+)	0	0						
Total n = 170	(−)	0	170	—	100.0	—	100.0	—	—
95% CI	—	—	—	—	97.8–100.0	—	—	—	—
Levofloxacin	(+)	29	3						
Total n = 57	(−)	4	21	87.9	87.5	90.6	84.0	87.7	0.74953 ($P < .0001$)
95% CI	—	—	—	71.8–96.6	67.6–97.3	76.9–96.6	67.4–93	76.3–94.9	0.57597 to 0.92308
Tetracycline	(+)	0	0						
Total n = 57	(−)	1	56	0.0	100.0	—	98.2	98.2	—
95% CI	—	—	—	0.0–97.5	93.6–100.0	—	98.2–98.2	90.6–100	—

(+), resistant; (−), sensitive; CI, confidence interval.