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CRHP Finder, a webtool for the detection of clarithromycin resistance in *Helicobacter pylori* from whole-genome sequencing data

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Abstract
**Background:** Resistance to clarithromycin in *Helicobacter pylori* (*H. pylori*) is mediated by mutations in the domain V of the 23S rRNA gene (A2142G, A2143G, A2142C). Other polymorphisms in the 23S rRNA gene have been reported to cause low-level clarithromycin resistance but their importance is still under debate. In this study, we aimed to develop and evaluate the CRHP Finder webtool for detection of the most common mutations mediating clarithromycin resistance from whole-genome sequencing (WGS) data. Moreover, we included an analysis of 23 *H. pylori* strains from Danish patients between January 2017 and September 2019 in Copenhagen, Denmark.

**Materials and Methods:** The CRHP Finder detects the fraction of each of the four nucleotides in nucleotide positions 2142, 2143, 2182, 2244 and 2712 of the 23S rRNA gene in *H. pylori* (*E. coli* numbering) by aligning raw sequencing reads (fastq format) with k-mer alignment (KMA). The nucleotide distribution in each position is compared to previously described point mutations mediating clarithromycin resistance in *H. pylori*, and a genotypic prediction of the clarithromycin resistance phenotype is presented as output. For validation of the CRHP webtool, 137 fastq paired-end sequencing datasets originating from a well-characterized strain collection of *H. pylori* were analyzed.

**Results:** The CRHP Finder correctly identified all resistance mutations reported in the sequencing data of 137 *H. pylori* strains. In the 23 Danish *H. pylori* strains, CRHP Finder detected A2143G (13%) in all resistant strains, and T2182C (13%) and C2244T (4.3%) nucleotide exchanges in only susceptible strains.

**Conclusion:** In this study, we present the validation of the first webtool for *H. pylori* resistance prediction based on the detection of 23S rRNA mutations (A2142C, A2142G, A2143G, T2182C, C2244T, T2712C) from WGS data of *H. pylori*.

**KEYWORDS**
23S rRNA gene, antimicrobial resistance, clarithromycin resistance, *Helicobacter pylori*, susceptibility testing, whole-genome sequencing
1 | INTRODUCTION

*Helicobacter pylori* (H pylori) represents a key factor in various gastrointestinal diseases. Empiric *H pylori* eradication treatment mainly consists of a triple therapy with a proton-pump inhibitor (PPI) combined with either the macrolide antibiotic clarithromycin or metronidazole and amoxicillin. Resistance to clarithromycin is a common cause of treatment failure in patients with *H pylori* infections. *H pylori* resistance to clarithromycin is often mediated by mutations in the domain V of the 23S rRNA, which is a structural element of the 50S large ribosomal subunit of bacterial ribosomes, inhibiting binding of macrolides to the 23S ribosomal subunit. In most cases, clarithromycin resistance can be attributed to the well-described mutations A2142G, A2143G, A2142C (E coli numbering). Other mutations in the domain V of the 23S rRNA gene, such as T2182C, G2224A, C2244T, and T2712C, have been reported to cause low-level clarithromycin resistance in some studies; however, their clinical relevance is still a matter of debate. Several research groups have published PCR methods for the detection of clarithromycin resistance mutations in the 23S rRNA gene of *H pylori*; however, they exclusively identify mutations at nucleotide positions A2142C, A2142G, and A2143G. WGS delivers a comprehensive description of resistance determinants present in a clinical isolate. Application of WGS on *H pylori* isolates to detect specific point mutations in the 23S rRNA allows the detection of hetero-resistance in *H pylori* strains or drug-resistant *H pylori* sub-populations that may be missed by phenotypic drug susceptibility. At the moment, there is no webtool available that allows easy and rapid analysis of whole-genome sequencing (WGS) data from *H pylori* strains and simultaneous identification of resistance mutations in the 23S rRNA gene. Considering this, we developed and evaluated the Clarithromycin Resistance *H pylori* (CRHP Finder) webtool for the detection of clarithromycin resistance conferring mutations A2142C, A2142G, A2143G, and additionally polymorphisms T2182C, C2244T, and T2712C with yet unknown resistance phenotype.

2 | MATERIALS AND METHODS

This study describes the development and validation of the free webtool CRHP Finder (available at https://cge.cbs.dtu.dk/services/CRHP-Finder/, and stand-alone: https://bitbucket.org/genomic epilemology/crhp-finder.git) using a previously well-characterized strain collection of 140 *H pylori* strains, from which 137 raw sequencing datasets were available for analysis. The current study includes an additional analysis of phenotypic and genotypic clarithromycin resistance data from 23 *H pylori* strains obtained from gastric biopsies of Danish patients with an indication for gastroscopy. The *H pylori* strains were collected between January 2017 and September 2019 at the Department of Clinical Microbiology in Rigshospitalet, Copenhagen, Denmark.

2.1 | Patients, gastric biopsies, and *H pylori* culture

Patients with dyspeptic symptoms who had a routine upper gastrointestinal endoscopy were included in the study. All twenty-three patients whose gastric biopsies were included in this study have given informed consent to participate. From the antral biopsies, *H pylori* culture was done on 7% defibrinated sheep blood (chocolate) agar plates at 37°C under microaerobic conditions (4% O2, 10% CO2, and 86% N2) for up to 7 days. Isolated *H pylori* strains were stored at −80°C until susceptibility testing, and WGS was performed.

2.2 | Phenotypic drug susceptibility testing

The minimum inhibitory concentrations (MICs) of clarithromycin were determined for the 23 *H pylori* isolates at the Department of Clinical Microbiology Copenhagen, Denmark. *H pylori* cultures were adjusted to a McFarland of 3, and phenotypic drug susceptibility testing was done on 7% defibrinated sheep blood (chocolate) agar plates using the clarithromycin E-test® (0.016-256 mg/L; bioMerieux). The agar plates were incubated under microaerobic condition for 3 days. The clinical breakpoint of clarithromycin was interpreted according to EUCAST.

2.3 | Identification of clarithromycin resistance mutations

Raw reads (fastq format) were aligned towards the 23S rRNA gene of *H pylori* using KMA (v1.2.14a), with the parameters: “-1t1 -cge -matrix.” To allow for different variants at each copy of the 23S rRNA, the nucleotide distribution of the target positions, 2142, 2143, 2182, 2244, and 2712, was examined using the assembly matrix provided by KMA. These steps were automatized in the program CRHP Finder, together with the found cutoffs to predict resistance for each mutation. The webtool gives a warning if the sequencing depth of 23S rRNA is below 100, and the depth is visible in the analysis output when using the webtool. The nucleotide distribution in each position is compared to previously described point mutations leading to clarithromycin resistance in *H pylori*, and a genotypic prediction of the clarithromycin resistance phenotype is presented as output. The webtool is designed to predict resistance (R) in relation to the presence of mutations A2142C, A2142G, A2143G, which are well known to cause resistance. The remaining mutations included in the webtool (C2244T, T2712C, and T2182C) are frequently described mutations but their importance in clarithromycin resistance is still unclear. Thus, as a start, the webtool was programmed to predict U (unknown) for these positions.
2.4 | Evaluation of the CRHP Finder

For validation of the CRHP Finder, a total of 137 FASTQ datasets (paired-end Illumina sequencing read files) from H pylori strains were submitted to the webtool. The 137 H pylori WGS datasets were obtained from the H pylori strain collection of the Institute of Medical Microbiology (IMM), University of Zurich, and collected between 2013 and 2017, and have been previously well characterized regarding their phenotypic and genotypic clarithromycin resistance.\(^1\) In addition, three clarithromycin resistant and 20 clarithromycin susceptible H pylori isolates were collected between January 2017 and September 2019 at the Department of Clinical Microbiology in Copenhagen, Denmark, and submitted to the CRHP webtool for analysis of their genotypic drug resistance.

2.5 | Data accession

Sequencing data from the study are available in the ENA database with project number PRJEB37266.

3 | RESULTS AND DISCUSSION

3.1 | Development and validation of the CRHP Finder

The CRHP Finder was validated by analyzing fastq datasets of 137 clinical H pylori isolates that have been previously well characterized in a study by Lauener et al\(^1\) regarding their phenotypic and genotypic clarithromycin resistance. When CRHP Finder analyzed the dataset, all previously reported mutations were detected. Initial attempts to use draft assembly data revealed the possibility of overlooking heterozygosity due to the presence of two copies of 23S rRNA in H pylori. Based on this finding using raw sequencing data are highly recommended when performing CRHP Finder analyses. In the validated dataset from Lauener et al\(^1\), clarithromycin resistance was exclusively related to the presence of mutations at positions 2142 and 2143 (A2142G, A2143G, A2142C) confirming previous findings.\(^4,5\) Polymorphisms at positions T2182 and C2244T were found in few of the susceptible strains except one strain with polymorphism T2182C which had an MIC of 1.5 (Table S1). However, Lauener et al\(^1\) describe how this specific strain was sent for retesting where an MIC of 0.5 mg/L was determined. Based on this result, and the fact that T2182C and C2244T were only present in resistant isolates when in combination with A2142G or A2143G we conclude that they most likely do not cause clarithromycin resistance, and the webtool was adjusted to predict “S.”

The mutations T2182C and C2244T were included in the CRHP Finder to further investigate their clinical importance. The webtool detected T2182C and C2244T in several of the 137 H pylori strains (Table S1). Since the Lauener et al study focused on reporting well-known resistance mutations in their study, all additional mutations were confirmed manually after analysis. The G2224A mutation described by Hao et al\(^11\) was initially included in the webtool but later created some issues. We found the G2224A mutation to be present in all resistant and susceptible H pylori strains included in our study (N = 160), and thus, it seems to be a naturally occurring polymorphism. Only a few of the sequences (0.16% - 1.52% of sequences) presented a low proportion of A instead of G at the 2224 position but the very low occurrence suggests that this deviation is due to sequencing error. Based on this, it was decided to exclude G2224A from the webtool. The remaining nucleotide exchange T2712C included in the webtool was not present in the 137 strains, and the predicted phenotype in CRHP Finder was therefore set to “U.”

3.2 | Analysis of 23 H pylori strains with CRHP Finder

The validated webtool was used to analyze clarithromycin resistance in our local strain collection of 23 H pylori strains. The results presented in Table 1 show clarithromycin resistance based on the A2143G mutation. Polymorphisms T2182C and C2244T were found in four of the susceptible strains, but none of the resistant strains. Polymorphism T2712C was not found in any of the 23 H pylori strains, and the predicted phenotype for T2712C therefore remains “U” for now.

Currently, antimicrobial therapy is mainly based on phenotypic drug susceptibility testing, but molecular-based methods, like quantitative polymerase chain reaction (qPCR), offer specific detection of resistance conferring point mutations in the 23S rRNA gene and can be directly applied on clinical specimens. Both qPCR and WGS can detect hetero-resistance in H pylori strains or drug-resistant H pylori subpopulations that may be missed by phenotypic drug susceptibility testing due to the very small and almost invisible colonies that H pylori forms. In this study, our results indicate that the commercially available qPCR assay (H pylori ClarRes Assay; Ingenetix, Vienna, Austria), which detects mutations A2142C, A2142G, and A2143G, would be sufficient to monitor clarithromycin resistance in clinical practice. When comparing qPCR assays to WGS a disadvantage of targeted molecular approaches is that they can only examine a limited number of nucleotide positions, while WGS delivers a more comprehensive description of resistance determinants present in a clinical isolate. Furthermore, the targeted assays may not cover new mutations potentially conferring drug resistance and thereby report false-negative results. In addition, the clinical relevance of new mutations can easily be assessed by retrospective analysis of WGS data, whereas targeted molecular assays would need to be remodeled and samples retested to overcome this. Since it is simple to add new positions to CRHP Finder and run a pre-existing dataset through the webtool, it offers a faster way to verify the clinical importance of suspected resistance conferring mutations. As outlook, we plan to use the webtool on our own national H pylori data to monitor the sufficiency of qPCR in detecting clarithromycin resistance conferring
mutations. The mutation database for CRHP Finder can be supplemented with newly arising clarithromycin resistance mutations, and since several polymorphisms beyond what has been mentioned in this paper have been described in the literature, the webtool provides an easy way for researchers to assess their clinical importance. Moreover, other researchers are able as well to do adjustments to the CRHP webtool by contacting the curator listed on the CRHP website.

In conclusion, we thoroughly validated the CRHP webtool to analyze raw sequencing reads from WGS for detection of common mutations in the 23S rRNA gene known to cause clarithromycin resistance in *H pylori*. CRHP Finder correctly detected all mutations reported in the previously characterized strain collection of 137 *H pylori* strains. By using the webtool, we can conclude that mutations C2244T, T2712C, and T2182C did not cause clarithromycin resistance in our tested strain collection as they were only found in susceptible strains or in combination with A2142G/A2143G. However, since the number of isolates carrying these mutations in our study were small, more analyses on C2244T, T2712C, and T2182C are needed before a final conclusion can be made on their importance in *H pylori* clarithromycin resistance. Evidently, the webtool can be used for future monitoring of the clinical relevance of new potential resistance conferring mutations in the 23S rRNA gene. It is available online for free and easy to use for individuals without advanced bioinformatics skills.

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TRANSPARENCY DECLARATIONS

None to declare.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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