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Testing the feasibility of using the common laboratory strain *E. coli* DH5α as a model system to study clarithromycin-resistance in *Helicobacter pylori*

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Antimicrobial-resistance (AMR) has emerged as a leading research field these days with an increasing number of drug-resistant microbes such as *Helicobacter pylori*. Most of the bacterial strains these days have evolved and developed resistance to a broad range of antibiotics of different classes resulting in AMR. *H. pylori* is the primary cause for the microbial infection-related gastric ulcers and sometimes cancers as well. Clarithromycin is a classical old generation antibiotic that lost its potency against the drug-resistant bacteria due to base substitutions in its target, the 23S ribosomal RNA. In this study we performed an extensive Bioinformatics analysis of the 23S rRNA sequence from *H. pylori* strains that are resistant to clarithromycin (Cly^r). In parallel we compared the 23S rRNA sequence of *E. coli* strains K-12 and DH5α that are commonly used in the laboratories with the wild type and mutant strains of *H. pylori* with a goal to evaluate Cly^r using the *E. coli* strains to circumvent the process of obtaining clinical patient samples of *H. pylori* which is often challenging administratively, ethically and legally. We evaluated the growth of DH5α strain in the presence of various concentrations of clarithromycin.

Keywords: *Helicobacter pylori*, gastric ulcers, DH5α, antimicrobial-resistance, clarithromycin-resistance.

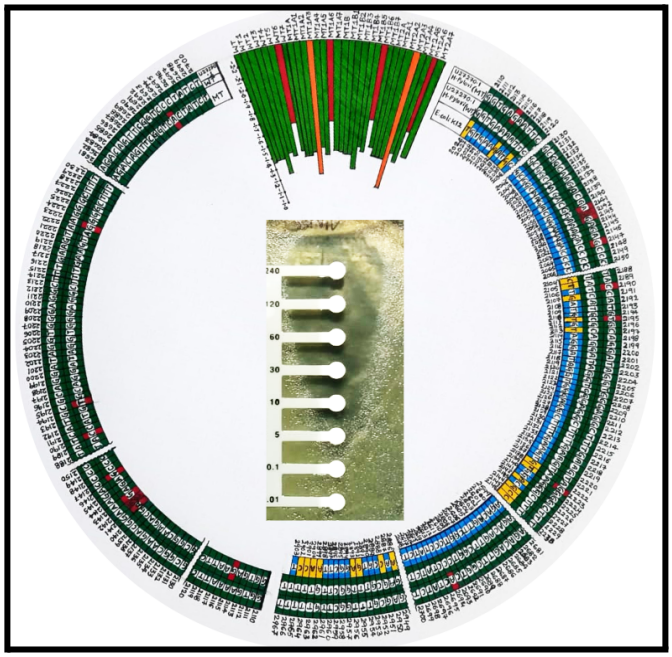

Figure 1. Circular plot showing the sequence alignment of *H. pylori* wild type vs. mutants (left semicircle) and *H. pylori* vs. *E. coli* (right semicircle). *H. pylori* mRNA stability for wild type vs. mutants (top of the circle). Clarithromycin activity discs in the middle are placed in the *E. coli* DH5α plate.

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Helicobacter pylori infected nearly more than 50% of the world population and it is mainly in developing countries (80%). In the past decade, the total number of cases was more than 4 billion. Gastritis, the inflammation of gastric mucosal lining could be acute or chronic. *H. pylori* has been known to cause non-erosive gastritis where the mucosal lining is still intact. However, the infection can be severe sometimes resulting in gastric ulcers and even cancer. Ulcers are formed generally in the stomach and proximal duodenum. *H. pylori* generally weakens the mucous coating of the stomach so that the inner layer is exposed to acid resulting in sores formation. These sores often lead to the formation of peptic ulcers. More than 60% of the gastric cancer cases are due to the infections by *H. pylori*.

Typically the current treatment regimens for *H. pylori* infections include combination of antibiotics (amoxicillin, clarithromycin, metroni -dazole, tetracycline, tinidazole, levofloxacin, rifabutin); acid production inhibitors (dexlan -soprazole, esomeprazole, lansoprazole, omepra -zole, pantoprazole, rabeprazole); bismuth subsalicylate and antihistamine drugs (cimeti -dine, famotidine, Pepcid, nizatidine). However, mutations in the drug targets of *H. pylori* lead to antibiotic-resistance. In the case of amoxicillin, mutations in the penicillin binding protein (PBP) lead to reduced accumulation of amoxicillin in the cell; mutations in the NADPH oxidoreductase lead to resistance against the DNA damaging agent metronidazole; due to mutations in the bacterial DNA gyrase levofloxacin-resistance occurs; either single or combination of mutations in the 16S rRNA of the ribosomal 30S subunit confer resistance against tetracycline while clarithromycin -resistance (Cly^r) arises due to mutations in the 23S rRNA.

In this study, we focused on evaluating the Cly^r using the commonly used laboratory strain of *E. coli*, DH5α. Clarithromycin is a semi -synthetic macrolide antibiotic that targets the bacterial 23S rRNA. Mutations with base substitutions such as A2142C, A2142G and A2143G in the 23S rRNA were primarily shown to be responsible for Cly^r in *H. pylori*. Other 23S rRNA mutations in *H. pylori* that are responsible in combination with the above mentioned are A2115G, G2141A, C2147G, T2190C, C2195T, A2223G and C2694A. Extensive sequence alignments were performed to evaluate the homology between the 23S rRNA molecules of *H. pylori* and *E. coli*, DH5α.

Materials & Methods:

NCBI Search: NCBI search was used for accession no: U27270.1 (23S rRNA and 5S rRNA of *Helicobacter pylori*) and also search for 23S rRNA of *E. coli* DH5-α (accession no: CP025520.1). The FASTA sequences from 372 to 3339 in *H. pylori* (accession no: u27270.1) and 3023394 to 3026311 in *E.coli* DH5α (accession no: CP025520.1) were identified as the coding sequence for 23S rRNA and were aligned using NCBI global align algorithm. Similarly the 23S rRNA sequence of *H. pylori* was aligned with the sequence of *E. coli* K-12 (PDB ID: 4V69) of 2903 length due to lack of a three-dimensional structure for *E.coli* DH5α 23S rRNA using NCBI global align algorithm.

RNA FOLD: Vienna RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) is a server-based open source software package that calculates the secondary structures of RNA sequences with a limitation of 7,500 nucleotides for partition function calculation and 10,000 nucleotides for minimum energy predictions. The wild type FASTA sequence (accession no: U27270.1) was used for building the RNA secondary structure and predicting its energy value. In order to download the image the online image converter (view in forna) was chosen in the download options. The image was then downloaded and saved in the PNG form.

Multiple sequence alignment: Clustal omega is the alignment software that helps to align three or more sequences up to align about 4000 sequences https://www.ebi.ac.uk/Tools/msa/clustalo/.

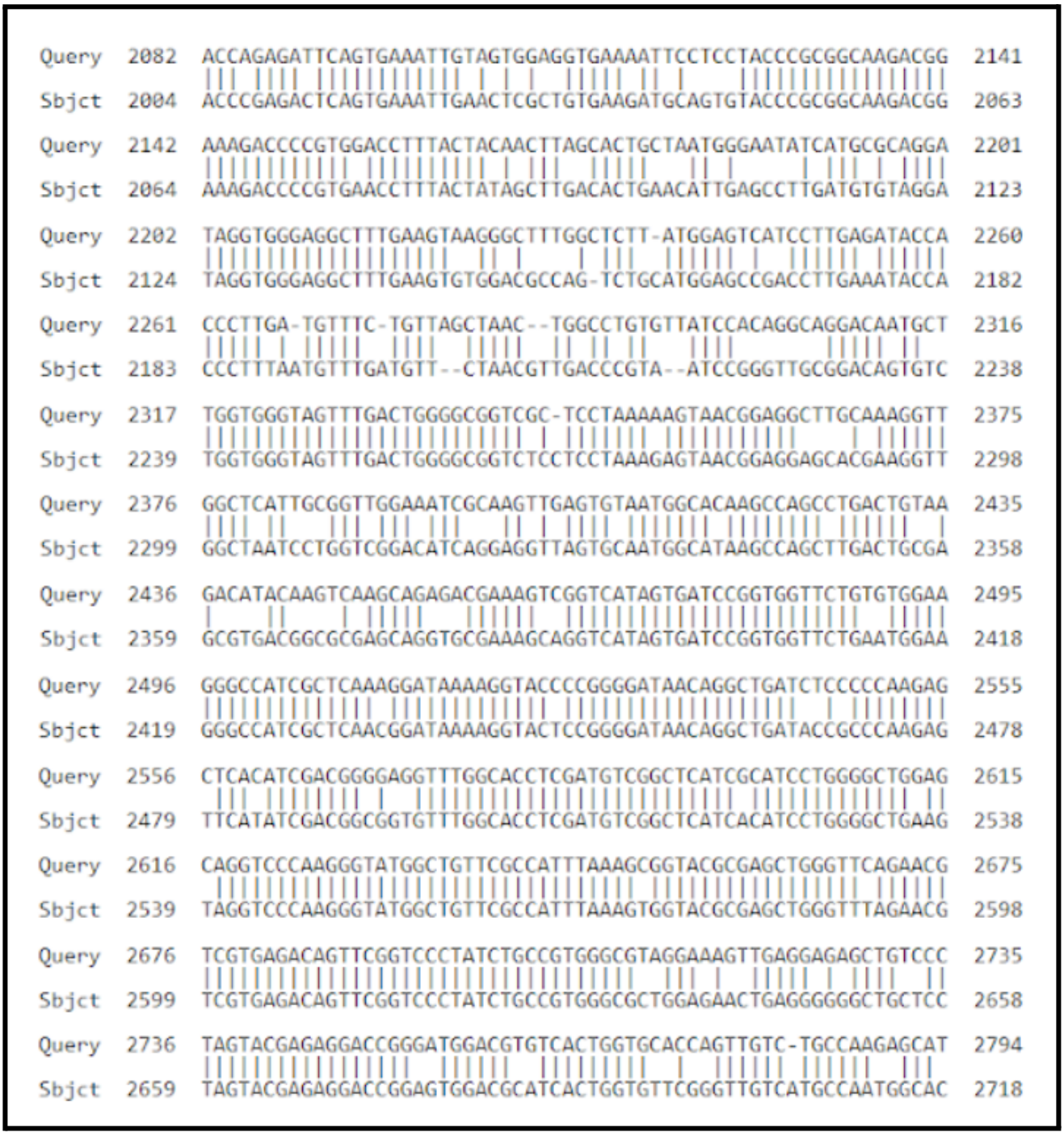

Figure 2. *H. pylori* vs. *E. coli* DH5α 23S rRNA alignment.

The sequence alignment was done for 23s ribosomal RNA sequences of *E.coli* DH5α and *H. pylori*.

Preparation of LB-Agar plates: In a clean bottle, 6.25gms of Luria Bertani powder and 3.75gms of agar powder were weighed to which 250ml of water was added and mixed well. Then it was autoclaved at 121℃, 15lbs pressure for 20minutes.

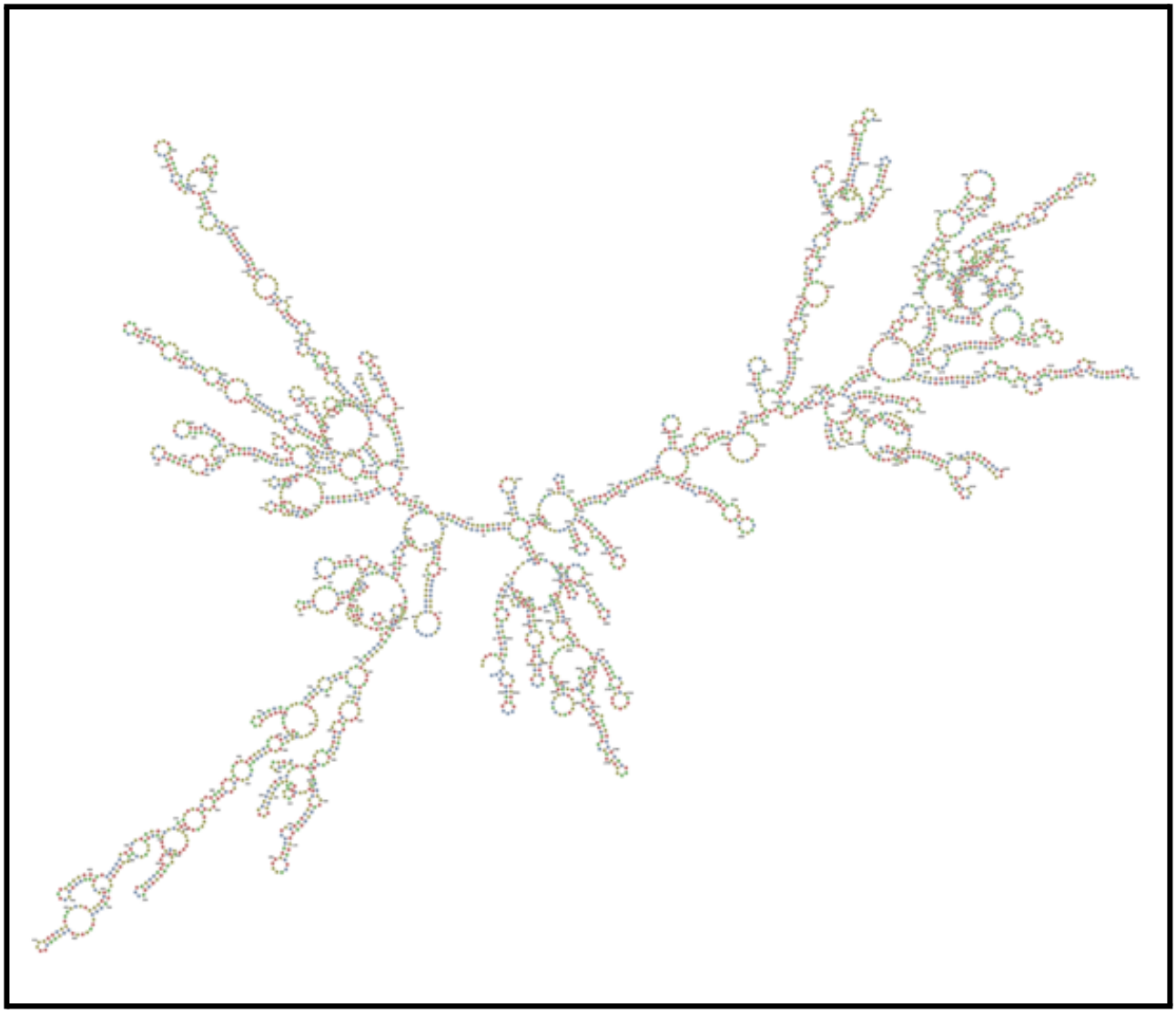

Figure 3. Predicted structure of *H. pylori* 23S rRNA.

The sterile petri plates were taken and media poured into them and allowed to solidify.

Antibiotic sensitivity test: Strips of clarithromycin of different concentrations were taken. Strip A has a concentration range of 0.01 to 240 micrograms and strip B of concentration 0.001 to 16 micrograms. *E.coli* DH5-α cell suspension was taken. Two prepared LB media plates were taken, 50 microliters of *E.coli* DH5-α cells were added to either plate. One LB plate was kept as negative control i.e., no addition of *E.coli*. Strip A of clarithromycin was placed in one plate and strip B in another plate. Incubate three plates at 37℃ for 16 hours.

Results and Discussion:

Bioinformatics: NCBI Search: To use *E. coli* DH5-α (accession number: CP025520.1) instead of *H. pylori* (accession no: U27270.1) for laboratory work their sequences are compared using global align to know their similarity (Figure 2). An overall 69% sequence homology was observed between the 2 which was considered as reasonable to consider the *E. coli* DH5-α as a model organism

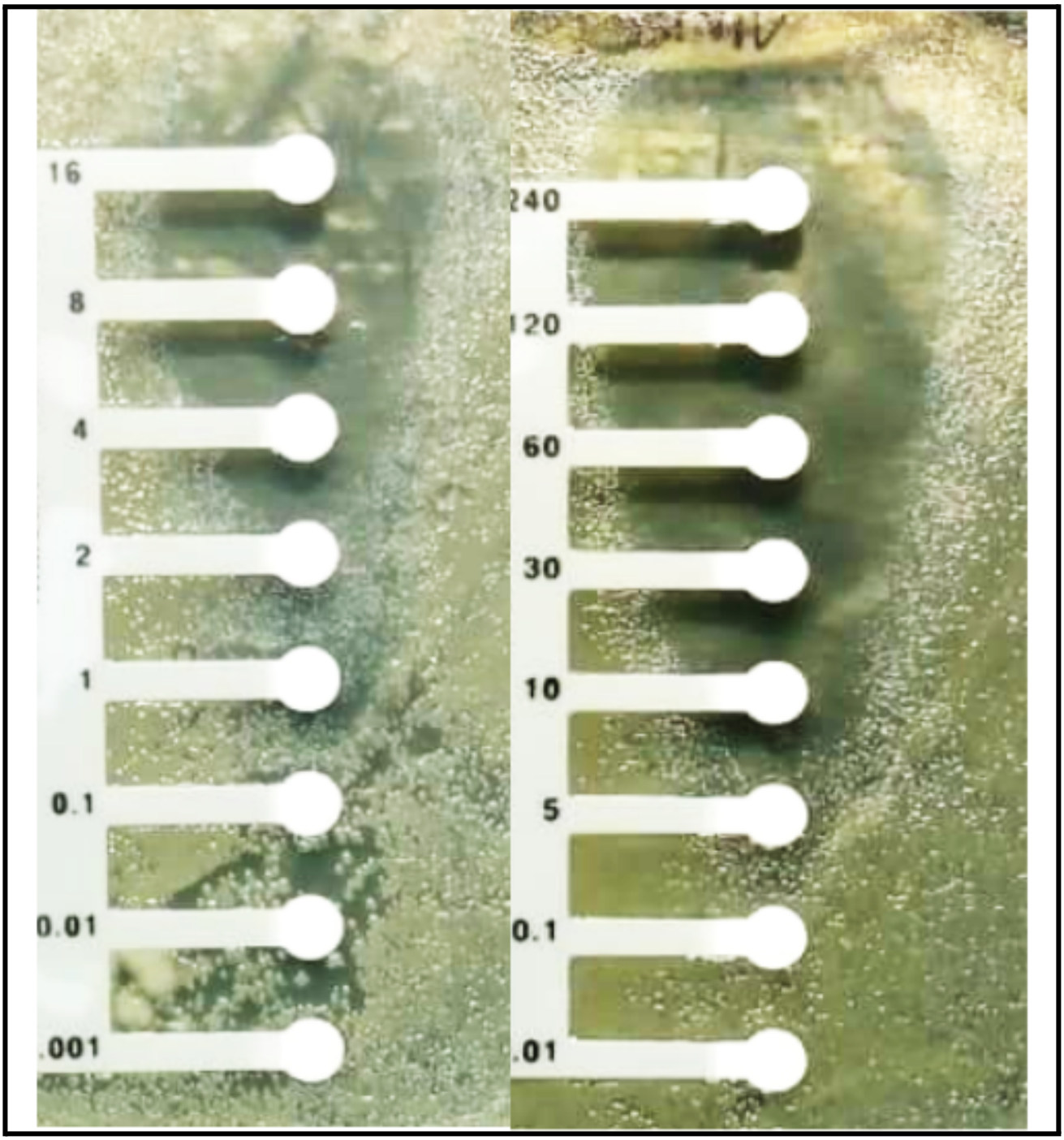

Figure 4. Clarithromycin sensitivity test for *E. coli* DH5α cells.

to study in the laboratory instead of *H. pylori* because *H. pylori* requires access to human clinical samples that require clearances from various departments such as ethical, clinical, research, etc. As shown in Figure 2, the overall sequence alignment looks good.

RNA secondary structures: Mutations in 23sribosomal RNA of *Helicobacter pylori* as

mentioned in introduction, mutations that provide resistance to clarithromycin in *H. pylori* are A2142G/C, A2143G, G2141A, C2147G, T2190C, C2195T ,A2223G, C2694A, A2115G. Secondary structures for all mutants including the wild type were predicted using RNAfold. Seven wild types are created by placing one mutation at a time in wild type sequence except A2142G/C and A2143G and they are labeled as WT1, WT2, WT3, WT4, WT5, WT6, WT7 and original wild type as WT. Taking A2142G, A2142C, A2143G as major mutations, remaining mutants were added one after one along with them, named as MT1A(A2142G), MT1B(A2142C), MT2A(A2143G). The mutations in MT1A(A2142G) group are MT1A1(A2142G, G2141A), MT1A2(A2142G, C2147G), MT1A3(A2142G, T2190C), MT1A4(A2142G, C2195T), MT1A5(A2142G, A2223G), MT1A6(A2142G, C2694A), MT1A7(A2142G, A2115G). The mutations in MT1B(A2142G) group are: MT1B1(A2142C, G2141A), MT1B2(A2142C, C2147G), MT1B3(A2142C, T2190C), MT1B4(A2142C, C2195T), MT1B5(A2142C, A2223G), MT1B6(A2142C, C2694A), MT1B7(A2142C, A2115G). The mutations in MT2A (A2143G) group are: MT2A1(A2143G, G2141A), MT2A2(A2143G, C2147G), MT2A3(A2143G, T2190C), MT2A4(A2143G, C2195T), MT2A5(A2143G, A2223G), MT2A6(A2143G, C2694A), MT2A7(A2143G, A2115G). The total number of mutations are 32. Minimum free energy, ensemble diversity and AMFE values of different mutations given by RNA Fold were plotted as shown in Figure 1. The overall AMFE values of the mutants are within the range of the wild type suggesting that these mutations are only affecting the clarithromycin-resistance but not the overall stability of the predicted secondary structures of the RNAs.

Clarithromycin activity test: This test was performed with the *E. coli* DH5-α strain of cells. The zones of inhibition for *E.coli* DH5-α cells to clarithromycin are given below:

[Clarithromycin]	Zone of inhibition
1 mcg	0.6cm
4mcg	2cm
8 mcg	1.7cm
16 mcg	2.8cm
120mcg	3.6cm
240mcg	3.9cm

Based on the zones of inhibition, we calculated the minimum inhibitory concentration of clarithromycin as 8 mcg. In future, this value will be compared to the mutant strains that show clarithromycin-resistance.

The current study focused on using the *E.coli* DH5-α cells as a model to study Cly^r seen in *H. pylori* human clinical samples. By establishing this model, one can speed up the process of studying the Cly^r using simple laboratory bacterial strains instead of going through complicated ethical, clinical and administrative guidelines and challenges involved. However, the complete model development is beyond the scope of this report and shall be continued in the future.

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Conflict of interest: This research article is an ongoing project currently at TCABS-E, Rajahmundry, India.