A Novel Reduction Strategy of Clarithromycin Resistance in *Helicobacter pylori*

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Background: Antibiotic resistance is a major therapeutic problem in patients infected with *Helicobacter pylori*. *H. pylori* clarithromycin resistant mutants have been evolved during antibiotic therapy, this is mainly due to 23s rRNA point mutations.
Objectives: In the present study, we investigated anti-mutational features of four traditionally Iranian medicinal plants on three local isolated *H. pylori* strains.
Materials and Methods: In this study clarithromycin resistance was used as a mutation indicator. Frequencies of such mutations in the presence and absence of plant extracts were evaluated. Mutation incidence was evaluated by Luria Delbruck fluctuation assay.
Results: The mean mutation frequency in *H. pylori* isolates was 27×10^{-9} which decreased at the presence of *Mirtus communis*, *Teucrium polium*, *Achillea millefolium* and *Thymus vulgaris* of plant extract, this amount was 97.4%, 95.2%, 63.7% and 19.6% respectively. Moreover, A-to-G transition at 2143 position (A2143G) was detected by PCR-sequencing as major point mutation causing clarithromycin resistant mutants.
Conclusions: The efficacy of these plant extracts in prohibiting resistance showed considerable results. This finding should be considered to use plant extracts with antibiotics to develop more effective eradication regimens.

Keywords: Medicinal Plants; *Helicobacter pylori*; *Achillea millefolium*; *Teucrium polium*; *Mirtus communis*; Clarithromycin; Mutation

1. Background

For the first time, *Helicobacter pylori* isolated from human gastric biopsies by Warren and Marshall in 1983 (1). Marshall reported that gastrointestinal disorders caused by self- ingestion of this bacterium (2). Today, this bacterium is known as the most common human pathogen affecting half of the world's population (3). The infections caused by *H. pylori* in human ranged from asymptomatic infections to symptomatic clinical presentations including acute or chronic gastritis, peptic ulcer diseases, gastric lymphoma and gastric adenocarcinoma (4). Eradication therapy is suggested for symptomatic patients. There are many different regimens used to treat *H. pylori* infections but, antibiotic resistance is the most important problems *H. pylori* eradication therapy (3, 5).

More than 60000 years ago, *H. pylori* colonized in human stomach and coevolved with humans since new human originated from his ancestors in Africa (6). Through the time *H. pylori* adapted to extraordinary acidic environment of gastric mucosa. The harsh environment of its niche enforces the bacterium to acquire particular mechanisms for rapid and effective adaptation to unfavorable conditions including presence of antimicrobial agents; as a result, *H. pylori* varied its gene pool. These variations were caused through point mutation, insertion, deletion or DNA exchange mechanisms (7).

There are some suspected mechanisms causing resistance against antibiotic agents but point mutation is accepted as the most important mechanism of antibiotic resistance in *H. pylori* (5). These sort of mutations occur at a very high frequency (10^{-5}-10^{-8}) in *H. pylori*, which is much larger than many other bacteria such as *Enterobacteriaceae* (8). Therefore, anti-mutagenic agents could be beneficial in reducing the occurrence of resistance-conferring mutations (9). In this respect, medicinal plants could be used as the potential source.

2. Objectives

In this study, we tried to evaluate anti-mutagenic properties of some plant extracts on the occurrence of mutations conferring clarithromycin resistance in *H. pylori*.

3. Materials and Methods

3.1. Plant Collection and Extraction

In the present study, four herbs which are used in traditional Iranian medicine, including *Teucrium polium* (ger-

mander), *Achillea millefolium* (yarrow), *Thymus vulgaris* (thyme) and *Mirtus communis* (myrtle) were purchased from a local medicinal herb shop. They were identified and confirmed by Professor G. H. Shahidi (Department of Botany, Faculty of Agriculture, Bahonar University, Kerman, Iran). The voucher numbers are kept at the Kerman University of Medical Sciences.

The seeds of *M. communis*, *T. vulgaris leaves*, flowers of *T. polium* and both leaves and flowers of *A. millefolium* were extracted as follows. The plant parts were washed, dried and grounded with a mechanical blender. Dry powders were soaked in extra pure ethanol (Merck, Germany) with solvent to sample ratio of 5:1 (v/w). After 5 days, each suspension filtered through Whatman filter paper No 5 (Whatman, England) and was concentrated, under reduced pressure. Each concentrated extract was evaporated to leave a solid pellet. The solid extracts were stored in dark at -20°C until use. Before application the solid extracts were dissolved in dimethyl solfoxide (DMSO, Merck, Germany) to a final concentration of less than 1%.

3.2. Bacterial Isolation

Three clarithromycin susceptible *H. pylori* isolates (K1, K7, and K11) were used in this study (see below). These isolates were obtained during a three-month-period from May to August 2011 from biopsy samples of patients referred to endoscopy unit of University hospital (Afzalipour, Kerman, Iran) in a previous study (10). All isolates were kept in brucella broth (BBL, USA) supplemented with 10% fetal bovine serum (Biochrom AG, Berlin, Germany) and 30% glycerol at -70°C.

3.3. Selection of Claritromycin Susceptible Isolates

Clarithromycin susceptibility was confirmed by inoculating a suspension of each isolate adjusted to a 3 of McFarland turbidity (approximately 9.0×10^8 CFU. mL^{-1}) on Muller-Hinton agar (Merck, Germany) plates containing 10% fetal bovine serum and 1 µg clarithromycin mL^{-1}. Clarithromycin sensitive isolates were not able to grow at such concentration (11).

3.4. Determination of Minimum Inhibitory Concentration

Final concentrations of 62.5, 125, 250, 500 and 1000 µg.mL^{-1} of the plant extracts were prepared for this purpose. Plant extracts were added to Muller-Hinton agar (Merck, Germany) enriched with 10% fetal bovine serum to give the above mentioned final concentrations. Plates were spot inoculated by 2 µL of a bacterial suspension adjusted to a turbidity 3 McFarland. The same plates without extract were also used as negative control. Incubation period was 3 days under micro aerobic atmosphere at 37°C (11).

The final concentrations of 0.03, 0.06, 0.125, 0.25, 0.5 and 1 µg. mL^{-1} of clarithromycin were also used for de-

termination the MIC of clarithromycin for each of these three isolates. Similar method and incubation condition were applied as above mentioned. Moreover, two concentrations (500 and 1000 µg.mL^{-1}) of each plant extract in combination with sub-inhibitory concentration (0.06 µg mL^{-1}) of clarithromycin in agar medium were used to evaluate the possible synergistic properties. Similar medium containing 0.06 µg. mL^{-1} clarithromycin without any plant extracts was also used as control.

3.5. Determination of Mutation Frequency

Mutation at 23s rRNA was introduced as the main cause of clarithromycin resistance in *H. pylori*. Therefore, in the present study we ignored clarithromycin. Mutation frequency was calculated using Luria Delbruck fluctuation assay by enumeration of observed mutant colonies grown from 20 parallel broth cultures in Brucella agar supplemented with 10% fetal bovine serum. Therefore, we prepared 25 independent tubes containing 1 mL Brucella broth supplemented with 10% fetal bovine serum. These tubes were inoculated with 10 µL of a preliminary culture (10^4-10^5 CFU. mL^{-1}) of each isolate and were incubated under micro aerobic condition for 3 days at 37°C. Five tubes from the above were used for enumeration of viable counts and 1 mL of each of the remaining 20 tubes was spread on agar media containing 1µg. mL^{-1} clarithromycin. After a 3-day- incubation, the colonies on the agar plates were counted. Mutation frequency was calculated from the proportion of median number of counted mutant colonies in 20 tubes divided by the total viable counts (12, 13). A 50-µL- sample from preliminary culture was plated on agar media containing 1 µg. mL^{-1} clarithromycin to confirm no preexisting resistant mutants.

3.6. Assessment of Mutation-Inhibiting Properties of Plant Extracts

In order to determine any anti-mutational properties of the plant extracts, mutation frequency was calculated in the presence of five sub-inhibitory concentration of plant extracts (62.5, 125, 250, 500 and 1000 µg.mL^{-1}). The results were compared with the experimental condition in absence of plant extracts. All the experiments were performed in triplicates.

3.7. DNA Extraction

DNA extraction performed using Bioneer genomic kit (Bioneer, South Korea) and the DNA extracted from all three isolates. Furthermore, DNA extracted by same method from one resistant mutant colony related to each isolate that was picked up after mutation frequency assessment.

3.8. PCR-Sequencing

A pair of oligonucleotide primers (Table 1) obtained from the known sequences of 23s rRNA gene of a wild

UA802 strain (GenBank accession No. U27270) were used to detect point mutations responsible for clarithromycin resistance. A 468 base pairs (bp) fragment corresponding to position 1811-2279 of the 23s rRNA gene was amplified in 35 cycles by a gradient thermal cycler (Biometra, Germany). PCR conditions were as follows, a primary denaturation at 94°C for 7 minutes followed by 1 minute denaturation at 94°C, 1 minute annealing at 57°C, 1 minute extension at 72°C and a final extension step for 5 minutes at 72°C as the end of amplification. The amplified fragments were sent to Macrogen Company (Gasan-dong, Geumchen-gu, Seoul, Korea) for sequencing. Point mutations were detected by comparing the sequences of the mutants (resistant colonies) with those of same fragment from 23s rRNA gene of a clarithromycin sensitive *H. pylori* strain UA802 in the GenBank, (accession No. U27270) (14).

Table 1. Oligonucleotide Primers

Primers	Sequences (5´→3´)	Position
HP-F	*TAAGGTGTGCCACAGCGAT*	1811-1830
HP-R	*CTAACAGAAACATCAAGGG*	2279-2260

4. Results

4.1. Extraction Yield

Extraction yield (% w/w) was calculated from the weight of final solid extract divided to the weight of the primary powdered plant parts. Our method have an extraction yield of 5% for *M. communis*, 7% *T. vulgaris* and *A. millefolium* to 9% for *T. polium*.

4.2. Minimal Inhibitory Concentration Values

The growth of all three isolates were inhibited at the concentration of 0.125 μg. mL^{-1} of clarithromycin and above. MIC of clarithromycin on mutated resistant colonies raised to more than 16 μg.mL^{-1}. Anti-*Helicobacter* properties of the plant extracts were evaluated at five different concentrations (62.5, 125, 250, 500 and 1000 μg.mL^{-1}). All of the applied extracts, except *M. communis* in the above mentioned concentration had shown no effect on the tested bacterial isolates. Therefore, it is expected that MIC of these extracts is more than 1000 μg.mL^{-1}. The MIC against *M. communis* was 1000 μg mL^{-1}. There was not any observed synergism between *M. communis* concentration less than 1000 μg.mL^{-1} and 0.06 μg. mL^{-1} of clarithromycin.

4.3. Point Mutation Conferring Resistance to Clarithromycin

DNA sequences of a 468-bp-fragment of the 23s rRNA gene from preliminary isolated bacteria (K1, K7, K11) compared with DNA sequences of clarithromycin sensitive wild UA802 strain. The results were completely identical; therefore preliminary isolated bacteria were preliminary confirmed as clarithromycin susceptible. A-to-G transition at 2143 position (A2143G) was detected as major point mutation in the sequences associated with the resistant mutant colonies incorporated to each of isolates.

4.4. Mutation Frequency of Clarithromycin Resistance

Mutation frequency was separately calculated for each isolate. Some minor differences between values were observed. The overall mean value was calculated to be 27×10^{-9}.

4.5. Mutation Frequency Values Applying the Plant Extracts

Results obtained from these experiments showed that at only three concentrations (250, 500 and 1000 μg mL^{-1}), the plant extract were effective in reducing the mutation frequency compared to the results obtained at the absence of plant extracts. Data collected after application of different concentrations of plant extracts were very similar (data not showed); therefore, we choose the minimal effective concentration at 250 μg mL^{-1} for the rest of experiments. Our findings showed a significant reduction in the mutation frequency value after application of extracts of *M. communis*, *T. polium* and *A. millefolium*, but, *T. vulgaris* extract was not as effective as the others. All three isolate were affected by these four plant extract with a minor differences.

The mean mutation frequency in the presence of extracts obtained from *T. vulgaris* and *A. millefolium* has been reduced to 21.7×10^{-9} and 9.8×10^{-9} respectively. In duplicated application of *M. communis* and *T. polium* extracts, we have not observed any mutant colony in agar media, though, bacterial cell suspension contained. 10^9 CFU.mL^{-1}. Therefore, bacterial suspension containing 10^{11} CFU.mL^{-1} was used in these cases. After inoculation of this bacterial suspension on the agar media containing *M. communis* and *T. polium* extracts the mean mutation frequency values were 0.7×10^{-9} and 1.3×10^{-9} respectively.

5. Discussion

Successful eradication of *H. pylori* usually resulted in resolving the symptoms of its infection; moreover, it may have resulted in preventing the possible gastric cancer. Different eradication regimens have been developed in this respect. In the recent decades, the clarithromycin-based triple therapy was used as the recommended first-line empirical treatment of *H. pylori* infections (3, 5). Antibiotic resistance, especially clarithromycin resistance, is significantly increased all over the world. Therefore it is no longer recommended to follow empirical therapy of *H. pylori* infections in areas with high resistance rates (3, 15, 16). Point mutations in 23s rRNA gene was described as the main mechanism of clarithromycin resistance in

H. pylori (17). The inhibition of these resistance-conferring mutations could serve as a novel strategy to overcome the antibiotic resistance issue. In recent years, a large number of literature reported the suggested anti-mutagenic agents in the medicinal plants (18-20).

Mutation is a complex process changing a genotype but not necessarily the phenotype. Therefore, the number of mutant colonies are significantly lower than the genotypically changed bacteria (11, 21, 22). We tracked the changes in the occurrence of mutation events which took place upon application of medicinal plants by means of "Mutation Frequency". Mutation frequency shows the actual consequence of this intervention, because only the favorable mutation resulted in the appearance of an antibiotic resistant phenotype are detected.

The results obtained by comparing the mean mutation frequency values in the presence and absence of plant extracts. It is shown that there are reductions in the mean mutation frequency values after application of *M. communis*, *T. polium*, *A. millefolium* and *T. vulgaris* this amounts were 97.4%, 95.2%, 63.7% and 19.6%, respectively. This was an interesting outcome which could explain the existence of certain mutation preventative potentials in these plant extracts. Our results reveal that the ethanolic extract from *T. vulgaris* was not so effective in reducing the mutation frequency of clarithromycin resistance. This is in contrast with the results of Wei and Shibamoto (23). Such differences may be attributed to essential oil effects in their experiments rather than the ethanolic extract which was used in our experiments. Aidi Wannes et al. reported weak anti-oxidant and probably anti-mutational activities of essential oils extracted from *M. communis* (24). They used essential oils obtained from leaf; stem and flower of this herb but ethanolic extract of the seeds was used in our experiment which showed its potent anti-mutational activity. These results demonstrated that the efficacy of ethanolic extracts is higher than that of essential oils. This finding was also supported by Aidi Wannes et al. (24). The results from previous studies reporting the anti-mutagenic activity and free radical-scavenging properties of these herbs supported our findings (18, 19, 25-29). The reduction effect of plant extracts on antibiotic resistance may justifies the usage of these medicinal plant extracts.

Some appropriate experiments under *in vivo* condition on animal models or human volunteers are needed to confirm these findings. In this respect, the medicinal plant derived substances in comparison to the chemical agents could obtain more patients compliance and ease in designing of clinical trials in human volunteers. The extracts obtained from the tested herbs, beside anti-mutational effects had reported anti-inflammatory and protective effect on gastric mucosal layer (30). Such valuable findings confirmed the beneficial use of those in traditional herbal medicine for treatment of gastrointestinal discomforts especially those caused by *H. pylori*. The accumulative results obtained from such investigations could lead to the development of more effective eradication regimens containing medicinal plants in combination with antibiotics. This may even be more beneficial if the actual effective ingredients from such plant extracts are recognized and the most suitable is to be added to the present antibiotic regimens.

Authors' Contributions

Omid Tadjrobehkar developed the primary idea and research protocol. Both of authors equally contributed in preparation of the manuscript.

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