

ORIGINAL ARTICLE

Persister cells as a possible cause of antibiotic therapy failure in *Helicobacter pylori*

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Author contribution: Parvin Bahmani collected the samples, performed the culture, and confirmed the isolates as *H. pylori* and also performed the antibiotic susceptibility testing. Sobhan Ghafourian performed the persister assay and edited the manuscript. Mina Mahmoudi interpreted the antibiotic susceptibility assay and edited manuscript. Abbas Maleki created all media and carried out the PCR. Nourkhoda Sadeghifard consulted on the project. Behzad Badakhsh performed the biopsy of patients and wrote the manuscript.

Abstract

Background and Aim: Due to the failure of antibiotic treatment and recurrence of infection in patients with *Helicobacter pylori*, this study was designed to find the possible cause of treatment failure and recurrence of the *H. pylori* infections in Ilam, Iran.

Methods: One hundred patients with specific symptoms of *H. pylori* infection were selected, and after taking a biopsy specimen, identification of *H. pylori*, antibiotic susceptibility assay, and persister cell assay were performed. In addition, after treatment, patients with persister cells were followed for possible recurrence of infection. Furthermore, an antibiotic susceptibility assay was performed.

Results: Our results demonstrated that, among 100 patients, 50% ($n = 50$) showed positive results for the existence of *H. pylori*. Among the susceptible isolates, 18% ($n = 9$) were persister cells that were sensitive to clarithromycin as confirmed by a 5 folds higher than the Minimal Inhibitory Concentration (MIC) of clarithromycin. The data were confirmed by following up the suspected patients.

Conclusion: Our results demonstrated that persister cells in *H. pylori* infections may be responsible to recurrent infection and antibiotic treatment failure. However, more research is needed to obtain more information in this area.

Introduction

Helicobacter pylori is one of the most substantial causes of bacterial infections commonly seen in human societies. As *H. pylori* is the fundamental reason for gastrointestinal disease, it is also the main reason for gastric cancer in humans.¹ In general, *H. pylori* causes a variety of gastrointestinal diseases such as gastric disorders, duodenal ulcer, peptic ulcer, adenocarcinoma, and gastric lymphoma.² There are several reports on the mortality of gastric lymphoma and peptic ulcer caused by *H. pylori*, which demonstrated considerable deaths around the world annually.³ On the other hand, chronic infections are also one of the very complicated disorders that cause duodenal ulcers and peptic ulcers in less than 10% of patients, as well as leading to cancer in less than 1% of patients.⁴ Therefore, control of these infections is extremely important even in the initial phases of the disease. As *H. pylori* has infected two-thirds of the world's human population, antibiotics should be prescribed more carefully.⁵

Unfortunately, lack of proper control of drug administration has led to a significant increase in antibiotic resistance.⁶ For this reason, the primary goal of the present study is to investigate antibiotic resistance in *H. pylori* in Ilam, Iran. According to the latest research in 2020, 51.2% of isolates were resistant to clarithromycin in Iran.⁷ Globally, *H. pylori* resistance to clarithromycin in Africa, Europe, Asia, and America is 50.8%, 25.1%, 24%, and 7.2%, respectively. Improper use of other macrolides such as azithromycin can be one of the reasons for resistance to clarithromycin, which is used to treat respiratory infections.⁸ In addition, metronidazole resistance has also been reported in 45% of clinical isolates in Iran.⁹ Subsequently, 16% resistance to amoxicillin and 12.2% resistance to tetracycline have been reported in Iranian clinical isolates of *H. pylori*.¹⁰

As *H. pylori* infection is very common, and antibiotic resistance is increasing sharply, and because the lack of treatment

for infection can endanger the patient's life, research in this area is extremely important.

H. pylori infection is highly common in humans, and failure to treat the infection can endanger the patient's life. Therefore, there is a great need for research in this area to control this libertine infection as soon as possible.

It should be noted that *H. pylori* can cause chronic infections that, in some cases, last for years and are even incurable.¹¹ One of the main causes of chronic infections is the persister cells, which develop very strong persistence to a particular antibiotic without any genetic justification. In summary, persister cells are dedicated to less than 1% of the bacterial population. After antibiotic treatment and elimination of nonpersister cells, persister cells remain and cause recurrence of the infection. This process can be repeated many times and creates an incurable infection (Fig. 1).^{12,13} As the persister cells can develop tolerance to antibiotics, the presence of these cells can be directly associated with antibiotic failure and causes of chronic disease.

Therefore, the present study was conducted to investigate the antibiotic resistance pattern and presence of persister cells in *H. pylori* collected from the patients from Ilam, Iran.

Method

Ethical approval. The members of the Ethical Committee of Ilam University of Medical Sciences, Ilam, Iran, approved the study (ID:1399.188).

Inclusion criteria. All patients with gastrointestinal symptoms and dyspepsia are referred to the gastrointestinal clinic in Ilam, Iran and are endoscopically treated by the gastroenterologist. *H. pylori* with endoscopic confirmation of tissue samples, positive rapid urease test or positive fecal antigen test were included in the study.

Exclusion criteria. Patients with underlying diseases such as liver cirrhosis, renal failure, severe heart disease, malignancy outside the gastrointestinal tract, age younger than 17 and over 80 years, taking steroids at the same time, had recent gastrointestinal bleeding, and/or were pregnant or breastfeeding were excluded.

Gastric biopsy sample collections and *H. pylori* phenotypic identification. Biopsies were obtained by the gastroenterologist in the gastrointestinal clinic in Ilam, Iran from 100 patients (samples were collected between September 2020 and December 2020) with symptoms of ache, burning pain in the abdominal area, burping, nausea, and bloating symptoms. Then, *H. pylori* was identified by histological observation, culture, and detection of urea enzyme by the Rapid Urease Test (RUT). Two biopsy specimens were taken from the antrum, and two biopsy specimens were taken from the corpus of each patient.

Phenotypic identification of *H.pylori*. Samples were transferred to a laboratory in a Stuart transfer medium (Merck, Lindenplatz, Germany).¹⁴ Endoscopic specimens were cultured rapidly or were refrigerated until culture. A portion of the sample was added to the Brucella agar (Merck, Germany) containing vancomycin (10 mg/L), trimethoprim (5 mg/L), amphotericin B (5 mg/L), polymixin (2500 U/L), 1% starch, and 5–7% sterile defibrillated blood sheep. The plates were stored at 37°C for 5–7 days with 10% carbon dioxide and 90–100% humidity; part of the sample was also homogeneous to do RUT. The positive result of the RUT was determined by changing the color from yellow to purple within 1 h. Other tests include catalase and oxidase and indole testing.¹⁵ The remaining homogeneous biopsy sample was used for gram staining.

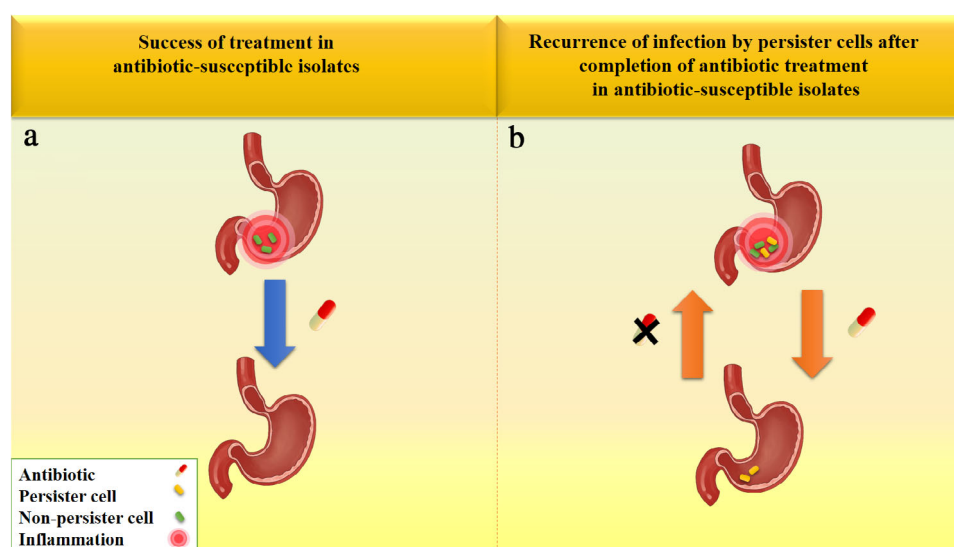


Figure 1 Antibiotic failure and recurrence of infection in persister cell and non-persister cell of *H. pylori* isolates. (a): In infected patients with antibiotic-susceptible isolates, which are not able to form persister cells, the patients recover after antibiotic treatment. In fact, antibiotic treatment will be successful. (b) In contrast, in infected patients with antibiotic-susceptible isolates, which can form persister cells, antibiotic treatment fails. Indeed, in isolates that form the persister cell, the symptoms of the disease subside with the administration of antibiotics. However, by stopping antibiotics, persister cells will cause the disease to recur. So, it becomes an incurable infection. ●, Antibiotic. ●, Persister cell. ●, Non-persister cell. ●, Inflammation.

Molecular confirmation by PCR. Initially, DNA was extracted from isolates by the boiling method.¹⁶ Therefore, the purity and quantity of the DNA were checked by NanoDrop (Berthold, Bad Wildbad, Germany). Then, in order to confirm *H. pylori* isolates, the detection of *ureC* gene was detected by PCR with listed primers in Table 1. Polymerase chain reaction (PCR) reaction mixture was prepared in a total volume of 25 μ L, containing 8 μ L of deionized water, 12 μ L of master mix PCR, 2 μ L of primers (forward and reverse), and 3 μ L of DNA template. Amplification was performed in a Bio-Rad thermocycler. The PCR protocol for the detection of the desired genes is available in Table 2.

Antibiotic susceptibility assay. Susceptibility of *H. pylori* clinical isolates to clarithromycin (2 μ g), metronidazole (5 μ g), amoxicillin (10 μ g), and tetracycline (30 μ g) (HI Media Laboratories, Mumbai, India) was evaluated by the disc diffusion test. For disk diffusion purposes, *H. pylori* isolates were cultured on Mueller Hinton agar (Merck, Germany) containing 10% defibrillated sheep blood and were incubated for 72 h at 37°C under microaerophilic conditions. Then, a McFarland 3 turbidity standard was prepared by adding colonies into the brain heart infusion (BHI) broth (Merck, Germany). Afterward, it was inoculated into the Mueller Hinton agar, and antibiotic disks were placed. Finally, plates were incubated at 37°C for 3 days under microaerophilic conditions. Subsequently, an agar dilution test was performed based on the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Accordingly, serial dilution was performed by adding inoculum in each glass test tube. The range of final concentrations for each antibiotic were 1–4 μ g/mL for tetracycline, 2–64 μ g/mL for metronidazole, 0.25–1 μ g/mL for amoxicillin, and 0.125–1 μ g/mL for clarithromycin. All the powder antibiotics were obtained from Sigma Company, China.

Determination of persister cells. Isolates were cultured up to the stationary phase. Then, 1 mL of the cultured bacteria and 200 μ L of the lysis solution (Sigma Aldrich) were mixed. Afterward, it was vortexed for 10 s and set aside at room temperature for 10 min. A total of 200 μ L of lysozyme enzyme solution was added to the solution and incubated at 37°C and centrifuged at 200 rpm. The solution was cultured on Luria Broth (LB) agar media in several dilutions to determine persister cells.¹⁸ In order to confirm persister

cell detection, growth of isolation was evaluated at an MIC fivefold higher than that of clarithromycin. Positive growth was considered evidence of persister cells. This test was performed twice.

Following the process of the patient's treatment.

Treatment success was assessed by a gastroenterologist based on clinical symptoms including ache, burning pain in the abdominal area, burping, nausea, bloating, and *H. pylori* antigen test (SHPAT) after 1 month of treatment.

Statistical analysis. SPSS version 16 was used to evaluate the prevalence of antibiotic susceptibility and persister cell formation in *H. pylori*.

Results

Phenotypic and molecular characterization demonstrates a high rate of *H. pylori* prevalence.

The study population was selected from patients who had obvious symptoms of *H. pylori* infection including, ache, burning pain in the abdominal area, burping, nausea, bloating symptoms from the gastrointestinal clinic in Ilam, Iran. Of 100 patients, *H. pylori* infection was confirmed in 50% (n = 50) of all patients using historical observation, culture, and RUT. All the isolates also were tested for the *ureC* gene that confirmed the phenotypic assays.

Disturbing rates of antimicrobial resistance to clarithromycin and metronidazole in *H. pylori* isolates.

Unfortunately, our results demonstrated that the state of antibiotic resistance in *H. pylori* is extremely disturbing. Therefore, several isolates were resistant to first-line antibiotics, metronidazole, and clarithromycin. More precisely, all isolates (n = 50) were resistant to metronidazole, which can greatly disrupt the healing process. Afterward, 66% (n = 33) of isolates were resistant to clarithromycin, and 28% (n = 14) of isolates were resistant to tetracycline and amoxicillin (Fig. 2).

The significant presence of persister cells in *H. pylori* clinical isolates.

The results of the persister cell assay indicated a very strong persistence in *H. pylori* clinical isolates. In detail, among all isolates, 18% (n = 9) of them were

Table 1 Primers designed for PCR to detect *ureC*

Gene name	Sequence (5' - 3')	Product length (bp)	References
<i>ureC</i>	F: TCTGTCTGATTCGCTTTTCTG R: AAGCTCGCTAAAAACGACC	820	[17]

Table 2 PCR condition to detect *ureC*

No. of cycle	Time	Temperature	Definite	Step	Program
1	5 min	94°C	Initial denaturation	1	Prog.1
35	30S	94°C	Denaturation	1	Prog.2
	30s	54°C	Annealing	2	
	1 min	72°C	Extension	3	
1	10 min	72°C	Final extension	1	Prog.3

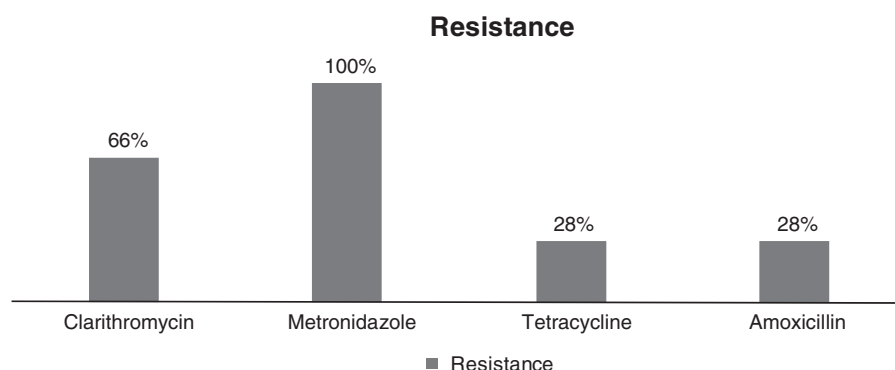


Figure 2 Antibiotic resistance pattern in *H. pylori* clinical isolates. The highest resistance was observed for metronidazole. ■, Resistance.

persister cells that were all sensitive to clarithromycin and at 5 folds higher than the MIC of clarithromycin.

Presence of persister cells in the recurrent cases of *H. pylori* infection. All patients were followed up, and the results were checked via the stool *H. pylori* antigen test (SHPAT) that performed in clinical laboratories. In nine patients who had persister cells, all were positive for SHPAT. Among the other 41 patients, only 2 patients showed SHPAT-positive results that were negative for persister cells.

These results suggested a potent relationship between persister cell formation and possible recurrent *H. pylori* infection.

Discussion

With the discovery of persister cells in 1994, a novel cause of the recurrence of infections has been presented.¹⁹ The consensus is that most populations of some bacterial isolates can disappear when faced with a specific antibiotic, but incredibly, some of them will remain, which can be revived if the antibiotic is removed.¹² Although the persister cells are not distinct from bacterial sensitive populations, they can cause a recurrence of the infection.¹² Therefore, persister cells are very valuable in the field of microbial infection treatment, such as infections caused by *H. pylori*.

Given that the genetic mechanism and formation of persister cells are unknown in *H. pylori*, more insight into practical solutions used to eradicate the persister cells requires more future studies. The present study reports the presence of a significant number of persister cells in the clarithromycin-susceptible *H. pylori* clinical isolates. However, the importance of persister cells lies in their amazing nature. In a significant number of patients, after receiving antibiotic treatment, the patient's symptoms decrease, but after stopping the antibiotic, the symptoms reappear. Surprisingly, this recurrence of the infection is seen in isolates that were sensitive to the antibiotic. In this study, this recurrence was also significantly seen in patients with symptoms of *H. pylori* infection, so we decided to evaluate the presence of persister cells in these patients.

The evaluation of antibiotic resistance can be very helpful in infection control and the treatment process. As there is no rapid laboratory test for the identification of antibiotic-resistant

H. pylori, physicians prescribe antibiotics empirically.²⁰ Accordingly, in this study, the antibiotic resistance pattern of the *H. pylori* clinical isolates was investigated.

Our results demonstrated that all *H. pylori* clinical isolates ($n = 50$) were resistant to metronidazole. The study by Setty *et al.* showed that 81% of *H. pylori* isolates were resistant to metronidazole²¹ and revealed a high rate of resistance similar to our study. In a meta-analysis study performed in Iran,¹⁰ among 21 articles published in different parts of Iran, the average resistance to metronidazole was approximately 61.6%, which was high. The high rate of resistance to metronidazole in Ilam may be because of the high rate of prescription of this antibiotic by physicians. In this study, resistance to clarithromycin was 66% ($n = 33$), which is not comparable with other parts of Iran. This resistance should be noted in Ilam, Iran. There is no perfect study to show the prevalence of *H. pylori* persister producers. We reported that persister cells may be responsible for the recurrence of *H. pylori* infection. Two patients showed the presence of *H. pylori* by SHPAT and negative results in persister assay, so, the failure of treatment may be explained by further analysis that these two patients were susceptible to clarithromycin and tetracycline.

Conclusion

This result probably explains the relationship between persister cell formation and the possible recurrence of infection. As failure to treat *H. pylori* infection can endanger patients' lives, identifying the causes of treatment failure is highly critical.

In addition, the high rate of existence of persister cells and unknown genetic pathways of persister cell formation are like an alarm for researchers to continue research in this field as fast as possible.

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