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spoT Gene Association with Biofilm Formation and Antibiotic Resistance in *Helicobacter pylori*

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Abstract: Most *Helicobacter pylori* (*H. pylori*) isolates carry *spoT* gene, which is the major gene of biofilm formation. Therefore, this research aimed to study the association of *spoT* gene with biofilm formation and antibiotic resistance in *H. pylori* isolates. Thirty-six isolates of *H. pylori* were studied for the presence of *spoT* gene by PCR method and their association with biofilm formation and antibiotic resistance. Biofilm formation of isolates was evaluated by microtiter-plate-based-method and, as well as, antibiotic susceptibility testing of isolates was evaluated by microtiter-plate method against antibiotics metronidazole, amoxicillin, tetracycline, and clarithromycin. Out of 33 *spoT*- positive isolates, 26 were able to form a biofilm, and 6 were not biofilm producers. None of 3 *spoT*- negative isolates were able to form biofilm. The frequency of resistance to metronidazole, amoxicillin, tetracycline, and clarithromycin in *spoT*+ *H. pylori* were 19 (57.5%), 12 (36%), 19 (57.5%), and 22 (66.66%), respectively. Biofilm formation and antibiotic resistance of *H. pylori* were associated with the harboring of *spoT* gene, so they could be served as genetic markers of antibiotic resistance. These results indicate the possible importance of *spoT* gene in the acquisition of resistance to antibiotics in this bacterium.

Keywords: *Helicobacter pylori*; antibiotic resistance; biofilm; *spoT* gene; Infection; Gastric disorder

1. Introduction

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterium with tropism in the human's stomach[1]. This bacterium is an etiological agent of different diseases, including gastritis, peptic ulcers, mucosa-associated lymphoid tissue lymphoma (MALT), and cancer in the gastric mucosa [2]. Gastric cancer (GC) is a common death-related cancer [2]. Therefore, treatment of *H. pylori* is important. The treatment regimens vary worldwide, according to drug availability and antimicrobial resistance. The level of *H. pylori* eradication has significantly eliminated and resistance to common antibiotics is the primary factor of this problem [3]. For instance, overuse of clarithromycin to treat respiratory tract infections has led to resistance to this antibiotic [4].

One of the reasons for preventing eradication therapy is biofilm formation in gastric mucosa. According to *in vitro* investigations, antibiotic resistance mutations in *H. pylori* happen in biofilms more than in planktonic cells. This information determined that biofilm formation of this pathogen may have a significant role in inhibiting and regulating its infections. Therefore, studying this characteristic in *H. pylori* could help in understanding the comprehensive mechanisms of infection and establishment [5].

H. pylori can change to coccoid form when exposed to antibiotics or unfavorable conditions[6]. One of the important factors for drug resistance is spoT gene which is expressed highly in the coccoid form[6]. SpoT enzyme responsibility is the synthesis and degradation of guanosine 3′-diphosphate 5′- triphosphate and guanosine 3′,5′-bispyrophosphate [(p)ppGpp]. It involves biofilm formation and controlling efflux pumps, i.e., proteins involve antibiotic removal and inducing resistance [7]. Indeed, recently has been found that the spoT gene in *H. pylori* thoroughly control its stringent response, mediating multiple important phenotypes[8].Therefore, current study, investigated the spoT gene associated with the biofilm formation and antibiotic resistance of clinical isolates obtained from subjects in Tabriz, Iran.

2. Materials and Methods

2.1. Patients.

Sampling was done from patients who underwent endoscopy from 2020/06/29 to 2021/05/15, at Imam Reza Hospital, Tabriz City, due to various dyspeptic diseases. The patients hadn't been using any antibiotics for 2 weeks prior to endoscopy. Diagnosis for patients was based on endoscopic observations and histology findings. The Regional Ethics Committee approved the project, Tabriz University of Medical Sciences, Iran (no: IR.TBZMED.REC.1398.1003, Date: 2019/12/23).

2.2. Growth condition of clinical isolates.

The thioglycolate broth was utilized as a transport medium for gastric biopsy specimens and shipped to the laboratory in cold temperature. The specimens were cultured on Brucella agar (Merck, Germany) containing 5% defibrinated sheep blood (Bahar Afshan, Iran), fetal bovine serum (FBS) (BTN, Iran), trimethoprim, vancomycin and amphotericin B (Merck, Germany). Cultures were placed at 37 °C for at least 5 days in a microaerophilic atmosphere (10% CO2, 5% O2, and 85% N2) provided by MART system (Anoxamat, Lichtenvoorde, Netherlands). *H. pylori* colonies were identified based on morphology, gram-staining, and urease tests. The colonies were kept at -70°C in Brucella broth medium containing 20% sterile glycerol until used.

2.3. Evaluation of biofilm formation.

To begin the experiment, wells of 96-well microtiter plates were filled with 200µl of Brucella broth supplemented with 7% FBS and 2% (w/v) yeast extract (Ibresco, Iran). Then, about 7µl bacterial suspension was inoculated into each well. The plates were placed under microaerophilic conditions at 37°C for 5 days. After that time, the contents of the wells were drained and washed three times with 180 µl of Phosphate Buffered Saline (PBS). To stabilize biofilms, 150 ml methanol was put for 20 min. After throwing away the methanol, crystal violet solution in water (0.1%) was used to stain biofilms for 20 min, then washed three times with

180 µl of sterile distilled water. The microtiter plates let to dry at room temperature. Then 220 ml of 33% acetic acid was added to each well to remove the biofilms. The optical density was read at 594 nm (OD594) by an ELISA reader. *Enterococcus fecalis* 29212 and Brucella broth without *H. pylori* served as positive control and negative control, respectively[9,10]. The biofilm was considered positive if the OD was more than the negative control. Experiments were performed twice at separate times (Figure 1).

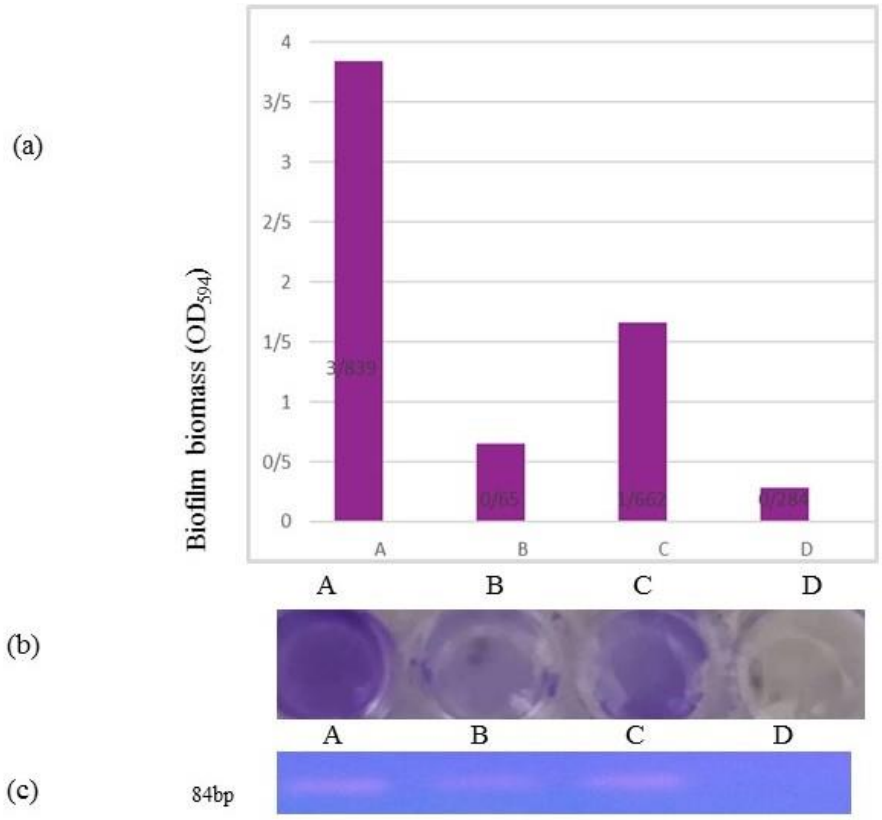

Figure 1. Biofilm formation by four *H. pylori* strains. **(a),** The graph shows quantification of biofilms formed after 5-days following culture in Brucella broth containing 7% FBS; **(b),** CV-stained biofilm of *H. pylori* strains grown in brucella-FBS broth; **(c),** genotyping of *spoT* gene by PCR, A, B, and C, *spoT* gene-positive *H. pylori* isolates, D, *spoT* gene-negative *H. pylori*

2.4. Minimum inhibitory concentration (MIC).

The antibiotic susceptibility profile of the isolates was performed by micro-broth dilution method against metronidazole, amoxicillin, tetracycline, and clarithromycin. Isolates with MIC >8 mg / L, > 0.5 mg / L, >4 mg / L, and >1 mg / L are considered resistant for metronidazole, amoxicillin, tetracycline, and clarithromycin, respectively. The bacterial suspension equivalent to 2.0 McFarland's standard was prepared from a 72-hour-old subculture of a blood agar plate in Brucella broth and diluted 100 times with the Brucella broth medium. The suspension (250µl) was added to 4.75 ml Mueller–Hinton broth supplemented with 7% FBS. A 100µl volume of the suspension was put into each well of a 96-well microplate, and finally, twofold serial dilutions of metronidazole, amoxicillin, tetracycline, or clarithromycin were added. The plates were placed at 37°C for 72 h in microaerophilic condition. Then, the plates were investigated for growth inhibition. The MIC was defined as the lowest concentration of an interesting antibiotic that completely inhibited visible bacterial growth.

2.5. Detection of spoT gene.

2.5.1. DNA extraction.

The suspension of *H. pylori* culture was prepared in saline. Deoxyribonucleic acid (DNA) was extracted by sodium dodecyl sulfate (SDS), proteinase K, and cetyl trimethyl ammonium bromide (CTAB) method [11] and stored at -20°C.

2.5.2. Conventional PCR assays.

Amplification of DNA was performed by thermocycler (Eppendorf). One pair of primers ureC was served for confirmation of isolates as *H. pylori*, which is present constitutively in *H. pylori*. The conditions of the primers (Metabion, Germany) served in this study are brought to Table 1. Each PCR reaction was done in a final volume of 20 µl as follows: *ureC*: 5 µl of 2· Hot Star Taq Master Mix, 3 µl of the DNA template, 1 µl of each primer [10 µM], and 10 µl of ddH2O. *spoT*: 5 µl of 2· Hot Star Taq Master Mix, 3 µl of the DNA template, 0.8 µl of each primer [10 µM], and 10.41 µl of ddH2O.

PCR products were analyzed using electrophoresis in a 1.5% (w/v) agarose gel in 1× Tris-borate-EDTA (TBE) buffer [7.5g EDTA, 55 g boric acid and 108 g Tris, pH = 8], at 100 V for 45 min. To stain the gels, ethidium bromide was used and photographed.

Table 1. PCR primers and conditions for detection of *ureC* and *spoT* genes

DNA region(s) amplified	Primer name	Sequences of primer (5′ to 3′)	PCR condition	Product (bp) size	Ref
spoT	Forward	CGCTTTAGAAATCAAACAGGTG	95°C, 1min; 35x (95˚C, 30s; 56˚C, 30s; 72˚C, 1min); 72˚C, 10min	84	[6]
	Reverse	AAATCCGTTACAATCGCAATG			
ureC	Forward	TGCTTGCTTTCTAACACTAACG	95°C, 1min; 35x (95˚C, 30s; 62˚C, 30s; 72˚C, 1min); 72˚C, 10min	355	[6]
	Reverse	TTGATGGCGATGCTGATAGG			

2.6. Statistical analysis.

All data were analyzed using SPSS 25 software. The comparison of the two groups was done using the Chi-square test. P value less than 0.05 was regarded as statistically significant.

3. Results and Discussion

3.1. Strain Collection.

In total, 200 gastric biopsy samples were obtained from educational hospitals in Tabriz, Imam Reza. Patients had different diseases, such as epigastric pain, dyspepsia, anemia, weight loss, and chest pain. The study was done on *H. pylori* isolates from gastric biopsy specimens of 36 patients (16 males and 20 females), with a mean age of 45 years, from 22 to 70 years old. In endoscopy 3 (8%) patients were diagnosed with duodenal ulcer disease, and 33 (92%) patients with non-ulcer diseases.

3.2. Distribution of biofilm formation and antibiotic resistance among H. pylori isolates.

In the current study, most of the isolates, 26 out of 36 (72%) isolates, could produce biofilm. The frequency of resistance was 21 (58.33%) to metronidazole, 15 (41.66%) to amoxicillin, 20 (55.55%) to tetracycline, and 25(69.4%) to clarithromycin. MIC ranges for metronidazole, amoxicillin, tetracycline, and clarithromycin were 0.25->32, ≤0.0625->32, 0.125->32, and 0.125- >32, respectively.

3.3. Association of biofilm formation and antibiotic resistance.

Out of 26 biofilm-forming isolates, 15 (57.6%) were resistant to metronidazole, 7 (27%) to amoxicillin, 13 (50%) to tetracycline, and 16 (61.5%) to clarithromycin. A significant relationship was observed between biofilm formation and resistance to antibiotics amoxicillin and tetracycline ($p < 0.05$).

3.4. PCR amplification of the ureC gene.

In order to amplify the *ureC* gene, primers *ure*C F and *ure*C R were used, and 355 bp product was obtained in all isolates. Primers were used as per described in Table 1. The protocol of the procedure is mentioned in Table 1.

3.5. Detection of the spoT gene and association with biofilm formation and antibiotic resistance.

The *spoT* gene was detected in 33 (91.6%) of 36 patients, 20 females and 13 males: 3 (100%) of 3 with duodenal ulcer disease and 30 (91%) of 33 with non-ulcer diseases. Out of 33 *spoT*- positive isolates, 26 were able to form a biofilm, and none of 3 *spoT*- negative isolates were able to form biofilm. Table 2 shows the association of *spoT* gene and biofilm formation in the clinical isolates. We found a significant relationship between *spoT* gene and biofilm formation ($p < 0.05$).

Table 2. Association of *spoT* gene and biofilm formation in 36 *H. pylori* isolates. There is a significant relationship between *spoT* gene and biofilm formation ($p < 0.05$).

	biofilm-forming isolates (26)	Non-biofilm-forming isolates (10)
spoT gene positive (33)	26/33(79%)	7/33(21%)
spoT gene negative (3)	0/3(0%)	3/3(100%)

Table 3. Comparison of antibiotic resistance in *spoT* gene positive and *spoT* gene negative of 36 *H. pylori* isolates. [a]

	***spoT*- positive (33), No. (%)**	***spoT*- negative (3), No. (%)**	**p-value**
MTZ resistance [a]	19 (57.5%)	2(67%)	> 0.05
AMX resistance [a]	12 (36%)	3(100%)	< 0.05
TET resistance [a]	19 (57.5%)	1(33%)	> 0.05
CLR resistance [a]	22 (66.66%)	3(100%)	> 0.05

Abbreviations: MTZ, metronidazole; AMX, amoxicillin; TET, tetracycline; CLR, clarithromycin

The frequency of resistance to metronidazole, amoxicillin, tetracycline, and clarithromycin in *spoT*$^+$ *H. pylori* were 19 (57.5%), 12 (36%), 19 (57.5%) and 22 (66.66%), respectively. Antibiotic resistance in *spoT* positive and *spoT* negative isolates are compared in Table 3. We found a significant relationship between *spoT* gene and resistance to amoxicillin ($p < 0.05$). In Table 4 distribution of the gene in the isolates based on antibiotics susceptibility patterns is shown.

Table 4. Distribution of *spoT* gene in 36 *H. pylori* isolates based on antibiotics susceptibility patterns.

	Resistant	Susceptible
Metronidazole	19/21(90%)	14/15(93%)
Amoxicillin	12/15(80%)	21/21(100%)
Tetracycline	19/20(95%)	14/16(87%)
Clarithromycin	22/25(88%)	11/11(100%)

There is little knowledge about the regulatory mechanisms of biofilm formation and antibiotic resistance in *H. pylori*. *SpoT* enzyme produces regulatory factors such as (p)ppGpp. It has been demonstrated that (p)ppGpp participates in biofilm formation and multidrug resistance. *SpoT* is also involved significantly in biofilm formation and multidrug resistance of *H. pylori* [12,13]. Poursin *et al*., in transcriptomic analysis, determined that *spoT* in coccoid forms of *H. pylori* is expressed 30 times higher than its spiral forms [7]. The *spoT* gene has a main role in antibiotic resistance, and its elevated expression in the coccoid form of the bacterium can be a useful diagnostic marker for identifying *H. pylori* during changes in its morphology [6].

Its association with biofilm formation and antibiotic resistance has been investigated in a few studies. Hence, we investigated the presence of the *spoT* gene, and its association with biofilm formation and antibiotic resistance. In the current study, 33 (91.6%) of 36 isolates harbored *spoT* gene. Out of 33 *spoT*- positive isolates, 26 were able to form a biofilm, and 6 were not biofilm producers. None of 3 *spoT*- negative isolates were able to form biofilm. We found a significant relationship between *spoT* gene and biofilm formation ($p < 0.05$). The present finding is supported by the findings of the previous studies regarding *H. pylori* [6,7,12]. In other bacteria such as *Vibrio cholera* (2012, in Texas, USA) [14] *Campylobacter jejuni* (2008, Canada) [15], it has been reported that this gene has an association with biofilm formation. Previous studies have demonstrated that the expression of *spoT* gene in its virulent phase is considerably prevented and is greatly lower than its expression in the coccoid form of the bacterium [6]. Moreover, recently, it has been found that *H. pylori spoT* gene thoroughly controls its stringent response, which subsequently mediates multiple phenotypes [8]. The stringent response, in turn, regulates the compatibility with nutrient deficiencies conditions, oxygen, and low pH, participates in the early step of the stationary phase in many bacteria, and causes a reduction in metabolic activity. The stringent response comprises a rapid accumulation of ppGpp and prevention of rRNA and tRNA synthesis [8,16]. As a matter of fact, ppGpp attaches to RNA polymerase and changes its affinity to different promoters like those associated with adaptation to stress conditions. It has an important effect on virulence gene expression in different pathogenic bacteria[6].

SpoT in *H. pylori* was indicated to control some efflux pumps [12,16] and subsequently affect biofilm formation and multidrug resistance [12,17]. It has been found that gluP participates in the biofilm formation and multidrug resistance of *H. pylori*, and the expression of gluP is mediated by *SpoT* [12]. *SpoT* controls a serum starvation response, which restricts growth and also maintains the helical morphology of the pathogen. At last, *SpoT* was determined to be substantial for intracellular macrophage survival during phagocytosis [18]. Biofilm production in microorganisms causes be less sensitive to drugs [19,20]. In this study, we determined the association of biofilm formation with antibiotic resistance in the isolates of *H. pylori*. There was a significant relationship between biofilm formation and resistance to antibiotics amoxicillin and tetracycline. The antimicrobial resistance of *H. pylori* to

metronidazole, clarithromycin, levofloxacin, and other antibiotics is elevating day by day since there isn't a standard application of antibiotics [21,22]. Moreover, *H. pylori* resistance to metronidazole has increased significantly in many regions of the world [23]. Therefore, *H. pylori* eradication has been recommended as the first-line treatment by current international guidelines.

In the current study, resistance rates to metronidazole, amoxicillin, tetracycline, and clarithromycin were 58.33% (21/36), 41.66% (15/36), 55.55% (20/36) and 69.4% (25/36), respectively. This study is supported by previous studies [24-31]. Additionally, *spoT* gene was demonstrated to be a global regulatory factor that has role in *H. pylori* antibiotic resistance by different mechanisms[16].

In these *spoT*$^{+}$ isolates, the frequency of resistance to amoxicillin, tetracycline, metronidazole, and clarithromycin was 12 (36%), 19 (57.5%), 19 (57.5%) and 22 (66.66%), respectively. We found a significant relationship between *spoT* gene and resistance to amoxicillin ($p < 0.05$).

A study in China (2016) revealed that *spoT* participates in the resistance of *H. pylori* to clarithromycin [16]. Also, in Japan (2006), Quinolone tolerance was reported to be correlated to this gene in *Pseudomonas aeruginosa* [32].

4. Conclusions

We concluded that the spoT gene is associated with biofilm formation and antibiotic resistance, so it could be served as a therapeutic target. Consequently, more studies should be undertaken to find the role of these genes in phenotypic characteristics. Accordingly, compounds could be produced to inhibit the expression of the spoT gene from eliminating antimicrobial resistance. Of course, these findings will shed light on the molecular mechanisms of antimicrobial resistance of *H. pylori*. Moreover, this information makes a new way to understand the functions of the spoT gene and its importance in the pathogenicity of *H. pylori*.

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Conflicts of Interest

The authors declare no conflict of interest.

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