Heliyon 10 (2024) e26809

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

CRISPR-like sequences association with antibiotic resistance and biofilm formation in *Helicobacter pylori* clinical isolates

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ARTICLE INFO

Keywords:
Helicobacter pylori
Antibiotic resistance
Biofilm
CRISPR-Like
Infection
Gastric disorder

ABSTRACT

Role of clustered regularly interspaced short palindromic repeats (CRISPR)-like sequences in antibiotic resistance and biofilm formation isn't clear. This study investigated association of CRISPR-like sequences with antibiotic resistance and biofilm formation in *H. pylori* isolates. Thirty-six of *H. pylori* isolates were studied for existence of CRISPR-like sequences using PCR method and their correlation with biofilm formation and antibiotic resistance. Microtiter-plate technique was utilized for investigating antibiotic resistance profile of isolates against amoxicillin, tetracycline, metronidazole and clarithromycin. Biofilm formation of isolates was analyzed by microtiter-plate-based-method. Out of 23 CRISPR-like positive isolates, 19 had ability of biofilm formation and 7 of 13 CRISPR-like negative isolates were able to form biofilm (P*value* = 0.445). In CRISPR-like positive isolates, 11 (48%), 18 (78%), 18 (78%) and 23 (100%) were resistant to amoxicillin, tetracycline, metronidazole and clarithromycin, respectively. Since CRISPR-like sequences have role in antibiotic resistance, may be applied as genetic markers of antibiotic resistance. But there was no substantial correlation between biofilm formation and existence of CRISPR-like sequences. These results indicate possible importance of CRISPR-like sequences on acquisition of resistance to antibiotics in this bacterium.

1. Introduction

Helicobacter pylori (*H. pylori*) is a curved pathogen that lives in the human's stomach [1]. This bacterium induces various diseases such as gastritis, peptic ulcers, mucosa-associated lymphoid tissue lymphoma (MALT), and cancer in the gastric mucosa [2]. Chronic

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https://doi.org/10.1016/j.heliyon.2024.e26809
Received 19 June 2023; Received in revised form 31 January 2024; Accepted 20 February 2024
Available online 21 February 2024

gastritis can develop to precancerous lesions including intestinal metaplasia and atrophic gastritis and eventually to the development of gastric adenocarcinoma [3]. Gastric cancer (GC) is a prevalent cancer that leads to death [2]. So, elimination of this pathogen is necessary. In order to eradicate *H. pylori*, different treatment regimens are utilized in the world, depending on drug availability and antimicrobial resistance. Eradication of the bacterium depends on novel issues such as metabolic changes and gut microbiota changes following treatment [4]. The rate of its eradication therapy has eliminated highly and resistance of the pathogen to most used antibiotics is main cause of this problem [5]. For example, treatment of upper respiratory tract infections with clarithromycin inordinately has caused resistance against this antibiotic [6].

A factor that exceed eradication therapy is existence biofilm on the surface of gastric mucosa. According to *in vitro* investigations, biofilm formation of *H. pylori* causes increasing of resistance to antibiotics, and mutations of antibiotic resistance are happened in biofilm more than in planktonic cells [7,8]. These findings showed that biofilm formation of this bacterium may have a substantial role in regulating of its infections. So, studying of biofilm formation in *H. pylori* could be useful in clarifying the comprehensive mechanisms of infection and establishment [9,10].

Point mutations on chromosome is cause of antibiotic resistance in the bacterium. CRISPR-Cas that is located in plasmids, bacteriophages or extracellular chromosomal DNA, is a protective system against foreign genetic elements [11]. The alteration of CRISPR-Cas systems in *Enterococcus fecalis* (*E. fecalis*) increases resistant to antibiotics [12,13]. In *H. pylori* genome there isn't functional CRISPR-Cas system, instead CRISPR-like loci have lately reported by *Bangpanwimon* et al. that are located in the vacA-like paralogue gene (VlpC, HP0922), and are correlated to colonization the stomach and resistance generation, showing that they may have a regulatory part [14,15]. These genes are assigned as *faaA* (flagella-associated autotransporter A), *imaA* (immunomodulatory autotransporter A), and *vlpC* (VacA-like protein C-Vlp C) [15].

The incorporation of the CRISPR-like and the VlpC gene makes the pathogen to be resistant to the host defense systems [12], and able to adjust to various stomach areas, allows to stick to the gastric epithelium [12]. Mutation in the *vlpC* gene in *H. pylori* is related to metronidazole resistance [15], but association of CRISPR-like system with antibiotic resistance isn't clear yet. Therefore, current study, investigated the CRISPR-like region associated with the biofilm formation and also antibiotic resistance of clinical isolates obtained from subjects in Tabriz, Iran.

2. Materials and methods

2.1. Patients

The research was done on patients who underwent upper endoscopy due to various dyspeptic diseases in Imam Reza and Shahid Madani Hospitals, Tabriz city. The patients hadn't received any drugs in order to treat *H. pylori* infection during 2 weeks prior to endoscopy. Endoscopic observations and histology findings were used to determine the diagnosis for each patient. Written informed consent including name, gender, age, chief complaint, clinical diagnosis, endoscopy, pathology, microbiology and PCR findings was gotten from patients who partook in this project and the research was confirmed by the Regional Ethics Committee, Tabriz University of Medical Sciences, Iran (No: IR.TBZMED.REC.1398.1003, Date: 2019/12/23).

2.2. Growth condition of clinical isolates

The gastric biopsy specimens were transmitted to laboratory into thioglycolate medium and homogenized on sterile slide and culture was performed on Brucella agar (Merck, Germany). *H. pylori* is a fastidious bacterium and grows in microaerophilic conditions. Supplements including 5% defibrinated sheep blood (Bahar Afshan, Iran), fetal bovine serum (FBS), trimethoprim, vancomycin and amphotericin B for inhibiting fungal growth were put on the medium. Plates were placed in microaerophilic atomosphere (5% O2, 10% CO2, and 85% N2) provided by MART system (Anoxamat, Netherlands), then incubated at 37 °C for up to 8 days. The bacterial colony morphology, urease and gram-staining tests were used for identifying isolates as *H. pylori* [16]. In order to store colonies at −80 °C, Brucella broth medium with 20% sterile glycerol were served.

2.3. Minimum inhibitory concentration (MIC)

The susceptibility of *H. pylori* isolates to amoxicillin, tetracycline, clarithromycin and metronidazole was determined by microbroth dilution method. According to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines isolates with MIC> 0.125 mg/L, >1 mg/L, >8 mg/L and >0.5 mg/L, are considered resistant to amoxicillin, tetracycline, metronidazole and clarithromycin, respectively. From a 72 h-old subculture, bacterial colony suspension equivalent to 2.0 McFarland's standard was prepared in Brucella broth and diluted 100 times with the Brucella broth medium. The suspension (250 μl) was put into 4.75 ml Mueller–Hinton broth supplemented with 7% FBS. A 100 μl volume of the suspension was added to each well of a 96-well microplate, and finally twofold serial dilutions of amoxicillin, tetracycline, clarithromycin or metronidazole were added. The plates were placed in 37 °C incubator for 72 h under microaerophilic atmosphere. After the time, growth inhibitions in them were observed. The lowest concentration of an antibiotic that entirely prevented bacterial growth verified as MIC [17].

2.4. Evaluation of biofilm formation

Briefly, each well of 96-well microtiter plates was filled with 220 μl of Brucella broth containing 7% FBS and 2% (w/v) yeast extract

(ibresco, Iran). Then each well was inoculated with approximately 7 μl bacterial suspension. The plates were placed under micro-aerobic atmosphere at 37 °C incubator for 5 days without shaking. Then all contents of the wells were slowly drained and by Phosphate Buffered Saline (PBS) were washed. For stabilizing the biofilms, 150 ml methanol was used for 20 min, afterward methanol threw away [18]. Crystal violet solution in water (0.1%) for 20 min was applied for staining biofilms, and then washed three times with 180 μl of sterile distilled water [19]. The microtiter plates were dried in room temperature. From 220 ml of 33% acetic acid was utilized for removing the biofilms to assay its formation. Then, the optical density was measured at 594 nm (OD594) by using a microtiter plate reader. *E. fecalis* 29212 was served as positive control and Brucella broth without *H. pylori* used as a negative control. The biofilm formation was determined by comparison OD of sample with than that of the control. Experiments were done twice at separate times.

2.5. Detection of CRISPR-like region

2.5.1. DNA extraction

Two loop-full of cultured *H. pylori* were suspended in saline. Deoxyribonucleic acid (DNA) was pulled out by proteinase K, sodium dodecyl sulfate (SDS), and cetyl trimethyl ammonium bromide (CTAB) method [20], and kept at −20 °C.

2.5.2. Conventional PCR assays

Thermocycler (Eppendorf) was used for DNA amplification. One pair of oligonucleotide primers including ureC was chosen for confirmation of *H. pylori* isolates, which is expressed constitutively in *H. pylori*. Table 1 shows conditions of the primers (metabion, Germany) used in this project. The final volume of each PCR reaction was 20 μl as follows: *ureC*: 3 μl of the DNA template, 5 μl of 2· Hot Star Taq Master Mix, 10 μl of ddH2O and 1 μl of each primer [10 μM]. CRISPR-like: 2 μl of the DNA template, 6 μl of 2· Hot Star Taq Master Mix, 10.4 μl of ddH2O and 0.8 μl of each primer [10 μM].

For analyzing of PCR products was used from electrophoresis in a 1.5% (w/v) agarose gel in 1 × Tris-borate-EDTA (TBE) buffer [108 g Tris, 55 g boric acid and 7.5g Ethylenediaminetetraacetic acid (EDTA), pH = 8], at 100 V for 45 min. Ethidium bromide was used for staining the gels and then photographed.

2.6. Statistical analysis

SPSS 25 software was applied for interpreting data. Two groups were compared using Chi-square test. P value less than 0.05 was supposed statistically significant.

3. Results

3.1. Strain Collection

Totally, 259 gastric biopsy samples were gathered from two educational hospitals in Tabriz, Imam Reza and Shahid Madani. Patients referred to endoscopy ward, suffering from epigastria pain, dyspepsia, anemia, weight loss and chest pain.

H. pylori isolates were recovered from gastric biopsy specimens of 36 patients (20 females and 16 males), with a mean age of 45 years, ranging from 22 to 70 years old, who underwent endoscopy from 2020/06/29 to 2021/05/15. Endoscopy diagnosis was reported as following: 3 (8%) patients 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 this study, 26 out of 36 (72%) isolates showed biofilm formation, but 10 (28%) isolates weren't able to form biofilm. OD of biofilm forming isolates was between 3.839 and 0.615.

The susceptibility of 36 *H. pylori* isolates to the antibiotics is brought in Table 2. The prevalence of resistance was 18 (50%) to amoxicillin, 20 (55.55%) to tetracycline, 21 (58.33%) to metronidazole and 25 (69.4%) to clarithromycin.

3.3. Association of biofilm formation and antibiotic resistance

Out of 26 biofilm forming isolates, 7 (27%) were resistant to amoxicillin, 13 (50%) to tetracycline, 15 (57.6%) to metronidazole and 16 (61.5%) to clarithromycin. There was significant association between biofilm formation and resistance to amoxicillin and

Table 1
PCR primers and conditions for detection of *ureC* and CRISPR locus.

DNA region(s) amplified	Primer name	Sequences of primer (5′–3′)	PCR condition	Product size (bp)	Ref
CRISPR locus	Forward	ATGGGGGCTTTAGTTTCAG	95 °C, 1min; 35x (95 °C, 30s; 52 °C, 30s; 72 °C, 1min); 72 °C, 10min	300	[15]
	Reverse	TAGCAAAAGGCGAACTTGA			
ureC	Forward	TGCTTGCTTTCTAACACTAACG	95 °C, 1min; 35x (95 °C, 30s; 62 °C, 30s; 72 °C, 1min); 72 °C, 10min	355	[21]
	Reverse	TTGATGGCGATGCTGATAGG			

Table 2
MIC by micro broth dilution method.[a] MIC—the lowest concentration at which the antibiotic did not show any visible growth of *H. pylori*.

Antimicrobial agent	MIC[a] ($\mu g\ ml^{-1}$)											MIC range
	≤0.0625	0.125	0.25	0.5	1	2	4	8	16	32	>32	
Amoxicillin	6	6	3	6	0	0	6	3	0	3	3	≤0.0625->32
Tetracycline	0	8	0	0	8	0	0	0	2	0	18	0.125->32
Metronidazole	0	0	6	0	0	9	0	0	3	0	18	0.25->32
Clarithromycin	0	2	3	6	0	0	0	0	12	02	11	0.125- >32

tetracycline ($p < 0.05$), but there wasn't significant relationship between biofilm formation and resistance to metronidazole and clarithromycin ($p > 0.05$).

3.4. PCR amplification of the ureC gene

By using primers *ure*C F and *ure*C R to amplify the *ureC* gene, the required PCR product of 355 bp was obtained in all isolates.

3.5. Detection of the CRISPR-like region and association with biofilm formation and antibiotic resistance

The CRISPR-like region was detected in 23 (63.8%) of 36 patients: 0 (0%) of 3 with duodenal ulcer disease, and 23 (70%) of 33 with non-ulcer diseases. The distribution of the CRISPR-like region in males was higher than females (16 males and 7 females). Out of 23 CRISPR-like positive isolates, 19 could form biofilm and 7 of 13 CRISPR-like negative isolates were positive for biofilm forming. Table 3 shows association of CRISPR-like region and biofilm formation in the clinical isolates. Significant relationship between CRISPR-like region and biofilm formation wasn't found.

Among CRISPR-like positive isolates, 11 (48%), 18 (78%), 18 (78%) and 23 (100%) were resistant to amoxicillin, tetracycline, metronidazole and clarithromycin, respectively. Antibiotic resistance in CRISPR-like positive and CRISPR-like negative isolates are compared in Table 4. We observed a significant association between CRISPR-like region and resistance to tetracycline and metronidazole ($p < 0.05$), but significant association between CRISPR-like region and resistance to amoxicillin and clarithromycin wasn't observed ($p > 0.05$). In Table 5 distribution of CRISPR-like region in the isolates based on antibiotics susceptibility patterns is shown.

4. Discussion

CRISPR-Cas technology, called adaptive immune system in bacteria, has various functions, including genome editing, manipulation of gene expression, bacterial virulence and also typing bacterial diversity [22–24]. But its association with antibiotic resistance has reported in few studies and its involvement in biofilm formation hasn't been studied. So, the part of the system in biofilm formation and antibiotic resistance of *H. pylori* is evaluated in this project by phenotypic and genotypic techniques.

The findings of this study determine that the CRISPR-like system participates in antibiotic resistance. The present finding is also in accordance with the results of the previous study [22].

A list of antibiotic-resistant "priority pathogens," has been published by the world health organization (WHO), tabulating 12 bacterial families that show the most threat to human health [25]; the list includes critical, high, and medium estates. Clarithromycin resistance in *H. pylori* has been classified in high estate. Additionally, *H. pylori* resistance to metronidazole has elevated to unacceptable level in many areas of the world [26]. So, *H. pylori* eradication was suggested as the first-line treatment by current international guidelines.

In our study, resistance degrees to amoxicillin, tetracycline, metronidazole and clarithromycin were 50%, 55.55%, 58.33% and 69.4%, respectively. Current study is in agreement with previous studies [27–34].

In current study the CRISPR-like region was present in 23 (63.8%) of 36 patients. For the first time, CRISPR-like sequences were detected by PCR in Thailand isolates genome, and were identified in all of the isolates of *H. pylori* [15]. In another study, CRISPR-like loci were detected in 22 of 53 (41.5%) *H. pylori* strains with different geographical origins, determined in their genomes present multiple conserved CRISPR-like sequences [12]. In CRISPR-like positive *H. pylori*, 11 (48%), 18 (78%), 18 (78%), and 23 (100%), were resistant to amoxicillin, tetracycline, metronidazole and clarithromycin, respectively. We observed a significant relationship between CRISPR-like region and resistance to tetracycline and metronidazole, but there wasn't significant relationship between CRISPR-like region and resistance to amoxicillin and clarithromycin. As mentioned earlier, the *vlpC* gene is location of CRISPR-like sequences, and mutation in the *vlpC* gene is related to metronidazole resistance [35]. Locating CRISPR-like sequences into the *vlpC* gene, effect on

Table 3
Association of CRISPR-like region and biofilm formation in 36 *H. pylori* isolates. There isn't significant relationship between CRISPR-like region and biofilm formation. $P > 0.05$

	biofilm forming isolates (26)	Non-biofilm forming isolates (10)
CRISPR-like positive (23)	(83%)23/19	(17%)23/4
CRISPR-like negative (13)	(54%)13/7	(46%)13/6

Table 4
Comparison of antibiotic resistance in CRISPR-like positive and CRISPR-like negative of 36 *H. pylori* isolates.

	CRISPR-like positive (23), No. (%)	CRISPR-like negative (13), No. (%)	p-value
AMX resistance[a]	11 (48%)	4 (31%)	>0.05
TET resistance[a]	18 (78%)	2 (15%)	<0.05
MTZ resistance[a]	18 (78%)	3 (23%)	<0.05
CLR resistance[a]	23 (100%)	2 (15%)	>0.05

[a] Abbreviations: AMX, amoxicillin; TET, tetracycline; MTZ, metronidazole; CLR, clarithromycin.

Table 5
Distribution of CRISPR-like sequences in 36 *H. pylori* strains based on antibiotics susceptibility patterns.

	Resistant	Susceptible
amoxicillin	(73%)15/11	(57%)21/12
tetracycline	(90%)20/18	(31%)16/5
metronidazole	(86%)21/18	(33%)15/5
clarithromycin	(92%)25/23	(0%)11/0

bacterial susceptibility to the host defense mechanisms [15], and cause able to survive in various stomach areas, make easier to attach to the gastric epithelium [12]. The outcomes of studies in different bacteria are inconsistent. Our result is in agreement with earlier literature, very recently in Pakistan was reported that CRISPR-Cas system in *Salmonella enteritidis* is involved in antibiotic resistance [22]. Recently another study in China found that in *Salmonella* isolates there was association between CRISPR system and antibiotic resistance genes (ARGs), but not with antibiotics [36]. A genomic analysis in New Zealand has suggested various parts about the attainment of antibiotic resistance genes in different species for CRISPR-Cas systems. This study determined that in some species CRISPR-Cas is positively correlated with ARGs, e.g. in *Klebsiella pneumonia* strains containing CRISPR-Cas there was tetracycline resistance genes with higher numbers. In most of *Neisseria meningitides* with CRISPR-Cas there is tetracycline resistance. Also, in Vibrio cholera between some ARGs and CRISPR-Cas was determined positive association. In some species including, *Staphylococcus aureus*, *Streptococcus pneumonia*, *Enterococcus faecium* and *Burkholderia cenocepacia* there is few CRISPR-Cas, but acquired ARGs is common. In several species such as *Salmonella enterica*, *Yersinia pestis*, *Streptococcus agalactiae*, *Campylobacter jejuni* and *Shigella sonnei*, CRISPR-Cas exists simultaneously in almost all genomes with ARGs. Whereas, in multiple bacteria, the existence of CRISPR-Cas is negatively associated with ARGs, e.g. CRISPR-Cas with fewer antibiotic resistance genes had positive association in *Pseudomonas aeruginosa* strains. In *E. faecalis*, CRISPR-Cas was inversely associated with glycopeptide and beta-lactam resistance [37]. Also, previously in a study was found that in *E. faecalis* harboring CRISPR was inversely associated with antibiotic resistance [24,38].

Biofilm formation of bacteria is a phenomenon which makes them less sensitive to antibiotics, thus removing of infection becomes difficult [39,40]. So, this study, determined association of biofilm formation with antibiotic resistance in clinical isolates of *H. pylori*. Out of 26 biofilm forming isolates, 27% were resistant to amoxicillin, 50% to tetracycline, 57.6% to metronidazole and 61.5% to clarithromycin. There was substantial association between biofilm formation and resistance to amoxicillin and tetracycline. However a study showed that in *Klebsiella oxytoca* clinical isolates, biofilm production was not significantly associated with β-lactam resistance [41].

Out of 23 CRISPR-like positive isolates, 19 could form biofilm and 7 of 13 CRISPR-like negative isolates were positive for biofilm forming. In CRISPR-like negative isolates 46% weren't able to form biofilm, which in comparison with biofilm forming isolates wasn't high, so it shows that biofilm forming in CRISPR-like negative isolates isn't reduced. In statistical analysis wasn't found significant relationship between CRISPR-like region and biofilm formation. Our result is in contrast with previous studies. In *E. coli*, CRISPRi through targeting luxS gene inhibits the biofilm formation [42]. Cas genes in *P. aeruginosa* contribute in biofilm production [43]. Also, in *Salmonella,* cas3 gene involves in biofilm production [44]. Recently, Meng Nie et al. have reported that CRISPR deficiency in *S. agalactiae* leads to decreased biofilm formation [45].

The differences in the correlation between CRISPR system and antibiotic resistance and biofilm formation may have several reasons such as antibiotic type, bacterial species, host and region.

5. Conclusions

We concluded that the CRISPR-like sequences are associated with antibiotic resistance, so could have therapeutic usage. Accordingly, it's possible to plan and produce compounds in order to eliminate the antimicrobial resistance by prohibiting the expression of the CRISPR-like sequences. Of course, through these findings novel seeing about the molecular mechanisms of antimicrobial resistance of *H. pylori* will be provided. Moreover, by this information a novel path about comprehending the CRISPR-like sequences functions will be provided in pathogenicity of *H. pylori*. The current study for the first time reports the connection of CRISPR-like sequences with antibiotic resistance and biofilm formation in *H. pylori* isolates, that demonstrated basal data about the association of the CRISPR-like sequences with biofilm formation and antibiotic resistance, thus further analysis is required to determine inducing antibiotic resistance is done just by this system or other genes are also involved. Also, further studies are needed to evaluate correlation

between CRISPR-like sequences with biofilm formation. Also the molecular process to influence the antibiotic resistance in *H. pylori* by CRISPR-like sequences hasn't been understood.

Funding

Faculty of Medicine, Tabriz University of Medical Sciences, with reference number 63656 as Ph.D. dissertation belongs to Leila Yousefi. The study's Supervisor was Dr. Hossein Samadi Kafil and all experiments were done in the Microbiology laboratory of the Drug Applied Research Center, Tabriz, Iran.

Availability of data and material

Upon sending request to the corresponding author.

Code availability

Not applicable.

Ethics approval

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This research was approved by the Regional Ethics Committee, Tabriz University of Medical Sciences, Iran (No: IR.TBZMED.REC.1398.1003, Date: 2019/12/23).

Consent to participate

All participant filled consent to participation and forms are available, participation was voluntary and presented to local ethic committee and supervisor of the research project.

Consent for publication

Not applicable.

CRediT authorship contribution statement

Leila Yousefi: Writing – review & editing, Writing – original draft, Software, Methodology, Formal analysis. **Hiva Kadkhoda:** Writing – review & editing, Methodology. **Masoud Shirmohammadi:** Writing – review & editing, Investigation. **Seyyed Yaghoub Moaddab:** Writing – review & editing, Investigation. **Reza Ghotaslou:** Writing – review & editing, Supervision. **Tahereh pirzadeh:** Writing – review & editing, Supervision. **Javid Sadeghi:** Writing – review & editing, Supervision. **Mohammad Hossein Somi:** Writing – review & editing, Investigation. **Mohammad Ahangarzadeh Rezaee:** Writing – review & editing, Supervision. **Khudaverdi Ganbarov:** Writing – review & editing, Conceptualization. **Hossein Samadi Kafil:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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