


Antibacterial Effects of Disulfiram in *Helicobacter pylori*

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Background: *Helicobacter pylori* infection poses a risk of the occurrence of gastrointestinal diseases, such as gastric cancer. Its incidence rate is significantly reduced by eradication, and thereby, eradication therapy is generally performed. Disulfiram is an oral prescription drug mainly used for the treatment of alcohol dependence. In recent years, reports have been made on its anticancer and antibacterial effects, and thus, it has recently become an interesting subject. This study aimed to examine the antibacterial activity of disulfiram, investigate the presence or absence of its antibacterial activity on *H. pylori*, and determine whether it could be a new bactericidal drug against drug-resistant *H. pylori*.

Materials and Methods: Drug-sensitive strains of *H. pylori* and amoxicillin-resistant, clarithromycin-resistant, and metronidazole-resistant strains were used, and a growth inhibition test of *H. pylori* using disulfiram was performed. Furthermore, the expression of urease, vacuolating cytotoxin A (VacA), and CagA, the virulence proteins of *H. pylori*, was quantitatively analyzed using the Western blotting method. In addition, for *H. pylori* used in this study, the 16SrDNA sequence, a ribosomal gene involved in protein production, was analyzed to examine the presence or absence of gene mutation.

Results: Disulfiram suppressed the growth of 7 out of 12 *H. pylori* strains at 1 µg/mL, and no correlation was observed between their susceptibility/resistance to current eradication antimicrobial drugs and disulfiram resistance. Disulfiram reduced the expression levels of urease, VacA, and CagA proteins. *H. pylori*, which showed resistance to disulfiram, tended to have fewer gene deletions/insertions in the 16S rDNA sequence; however, no specific mutation was detected.

Conclusion: Disulfiram has a bactericidal effect on *H. pylori* at low concentrations, suggesting that it can be used as a supplement for current *H. pylori* eradication drugs.

Keywords: disulfiram, *Helicobacter pylori*, urease, vacuolating toxin, CagA

Introduction

Helicobacter pylori is a Gram-negative spiral bacterium that inhabits the human gastric mucosa. *H. pylori* infection poses a risk of the occurrence of gastrointestinal diseases, such as peptic ulcer, gastric mucosa-associated lymphoid tissue lymphoma, and gastric cancer,¹⁻⁴ however, its occurrence is significantly reduced by eradication.^{5,6} In Japan, eradication therapy using amoxicillin and clarithromycin is the primary treatment for *H. pylori* infection; however, in recent years, the eradication success rate is decreasing due to the increased incidence of drug-resistant *H. pylori*.⁷

Disulfiram is an oral administration drug marketed under the trade name Antabuse and used primarily indicated for the treatment of alcohol dependence.

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When disulfiram is orally ingested, one molecule of disulfiram is converted into two molecules of diethyldithiocarbamic acid in the gastrointestinal tract and blood and further metabolized into diethyldithiocarbamic acid methyl ester sulfoxide. When these metabolites react and bind to activation sites such as aldehyde dehydrogenase, alcohol metabolism is reduced, resulting in hangover symptoms, resulting in avoidance of alcohol inoculation.⁸ Disulfiram has been used as a therapeutic agent for alcohol dependence for many years; however, studies have re-focused on its anticancer^{9,10} and antibacterial activities in recent years.^{11,12}

Therefore, this study focused on the antibacterial activity of disulfiram, investigated the presence or absence of its antibacterial activity against *H. pylori*, and investigated whether it could be a new bactericidal drug against drug-resistant *H. pylori*.

Materials and Methods

Strains

H. pylori strains were isolated from patients admitted to Okayama University Hospital with informed consent. Ethical approval to carry out the study was obtained a priori from the Okayama University Ethics Committee (Number 2034). *H. pylori* samples were collected in accordance with the 1964 Helsinki declaration and its later amendments. E-test was used to measure susceptibility to amoxicillin, clarithromycin, and metronidazole for antibacterial sensitivity. If the minimum inhibitory concentration (MIC) value of amoxicillin is ≥ 0.1 $\mu\text{g/mL}$; clarithromycin, ≥ 2 $\mu\text{g/mL}$; and metronidazole, ≥ 1 $\mu\text{g/mL}$, the strain is identified as resistant to each antibiotic. Then, drug-sensitive, amoxicillin-resistant, clarithromycin-resistant, and metronidazole-resistant strains were selected. Table 1 shows drug resistance values of the selected 12 strains.

H. pylori Growth Inhibition Test with Disulfiram

Whether disulfiram suppresses the growth of *H. pylori* was measured. About 20 mL of brain heart infusion (BHI) medium containing 5% horse serum was placed in a 50-mL liquid culture flask, and the medium was allowed to stand in a carbon dioxide (CO₂) incubator overnight to saturate the liquid medium with CO₂. Disulfiram (Sigma-Aldrich) was added to a CO₂-saturated liquid medium to prepare a chemical medium with a disulfiram

Table 1 MIC Values of *H. pylori* Strain Used

Strains	Amoxicillin ($\mu\text{g/mL}$)	Clarithromycin ($\mu\text{g/mL}$)	Metronidazole ($\mu\text{g/mL}$)
S1	<0.016	<0.016	0.047
S2	<0.016	<0.016	<0.016
S3	<0.016	<0.016	0.016
AC1	6	16	0.32
AC2	0.75	1	256<
AC3	256<	256<	16
CH1	0.032	256<	256<
CH2	0.032	256<	256<
CH3	0.023	12	96
MZ1	<0.016	6	0.96
MZ2	0.023	0.75	128
MZ3	<0.016	0.25	48

Abbreviations: MIC, minimum inhibitory concentration; AC, amoxicillin-resistant strain; CH, clarithromycin-resistant strain; MZ, metronidazole-resistant strain.

concentration of 10, 5, 1, and 0 $\mu\text{g/mL}$. As a pre-culture, *H. pylori*, which had been pure-cultured on BHI agar medium containing 7% horse defibrin blood at 37°C for 4 days under microaerobic conditions, was suspended in a BHI solution containing 5% horse serum at OD₆₀₀ = 1.0 (10⁸ CFU/mL). *H. pylori* infused with 500 μL each suspended medium was inoculated and cultured by shaking at 37°C under microaerobic conditions for 4 days. Absorbance was measured at OD₆₀₀ nm every day, and the bacterial growth was confirmed by increased absorbance. Bacterial solution was collected after shaking the culture, and morphological changes of the bacteria were observed with Gram staining. A portion of the cultured medium was applied to the horse blood BHI agar medium and cultured at 37°C in microaerobic conditions for 7 days to confirm its effects on *H. pylori* developmental ability.

Protein Expression Level Measurement

The amount of *H. pylori* was estimated from strain absorbance in which the growth of *H. pylori* was confirmed in the growth inhibition test. When OD₆₀₀ = 0.5, the amount of bacteria is approximately 10⁷ CFU/mL. Bacterial solutions were collected from media with disulfiram concentrations of 0 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$ by centrifugation. Bacteria were re-suspended into distilled water with 1 mM of protease inhibitor phenyl-methyl-sulfonyl fluoride and disrupted by ultrasonic waves in ice for 10 min using a device (Bioruptor, Cosmobio). A sample was prepared by diluting the same amount as the SDS sample buffer and heating at 100°C for 10 min. About 10 of the prepared sample was added to 1

well, electrophoresed at 90 V for 2 h using the SDS-PAGE, and then transferred to a polyvinylidene fluoride (PVDF) membrane at 100 mA for 4 h. After the transfer, the PVDF membrane was blocked overnight with phosphate-buffered saline (PBS) containing 10% skim milk. After washing with PBS for 5 min and 3 times, urease (30 kDa) antibody (in house), CagA antibody (Funakoshi), or VacA antibody (Santa Cruz Biotechnology, Inc) diluted 1000-fold with 10% skim milk-added PBS were added as primary antibodies and shaken for 2 h; horseradish peroxidase-labeled antibody (Dako) diluted 1000-fold with 10% skim milk-added PBS was added as a secondary antibody and shaken for 1 h; and the color was developed by Pierce® Western Blotting Substrate (Thermo Fisher Scientific), and the band was detected and quantified by chemiluminescence using the Amersham Imager 600 (Cytiva).

Confirmation of Gene Mutation

DNA Extraction from the Bacteria

DNA was extracted from the strain according to the product protocol of QIAamp DNA Micro Kit (QIAGEN). The bacteria were suspended in a 1.5-mL Eppen tube with 500- μ L of physiological saline and centrifuged at 15,000 rpm for 2 min, and the supernatant was discarded. The remaining precipitate was pulverized by tapping or vortexing, added with 300 μ L of Nuclei Lysis Solution, and the mixture was suspended by pipetting, incubated at 80°C for 5 min, and cooled at room temperature. After adding 1.5 μ L of RNase and mixing by inversion, the mixture was incubated at 37°C for 15 min and cooled at room temperature. Thereafter, 100 μ L of protein precipitation solution was added, vortexed, and then cooled in ice for 5 min. Centrifugation was performed at 4°C and 15,000 rpm for 3 min, the supernatant was placed in a new Eppen tube, and 300 μ L of isopropanol was added. Thereafter, the mixture was centrifuge at 4°C and 15,000 rpm for 2 min, the supernatant was discarded, and 300 μ L of 70% ethanol was added. It was centrifuged again at 4°C and 15,000 rpm for 2 min, and the ethanol in the tube was carefully aspirated. After drying the tube at 37°C, 50 μ L of DNA rehydration solution was added and incubated at 65°C for 60 min.

Polymerase Chain Reaction Detection and Sequence

The sequence of 16S rDNA, a ribosomal gene involved in protein production, was detected by polymerase chain reaction (PCR) using primers 16S-F and 16S-R. The sequence of each primer is shown in Table 2.

Table 2 PCR Primers

Primer Name	Sequence	Product (bp)
16S-F	5-CTGAGACACGGTCCAGACTC-3	1055
16S-R	5-CAAGACCCGGGAACTGATTTC-3	

Using the DNA of the extracted bacteria as a template, PCR was performed in a total amount of 50 μ L. TaKaRa Ex Taq (5 units/ μ L) of 0.5 μ L; 10 \times Ex Taq Buffer, 5.0 μ L; dNTP mixture (2.5 mM each), 4.0 μ L; template, 1.0 μ L; primer pair, 1.0 μ L; sterile distilled water, 38 μ L were mixed. PCR conditions were 98°C for 1-min denaturation, 98°C for 10 s, 60°C for 30 s, and 72°C for 30 s for 35 cycles. A 5- μ L PCR product was electrophoresed on a 2% agarose gel for 30 min. The gel after migration was stained with ethidium bromide for 20 min and observed by ultra-violet irradiation using an electrophoresis gel imaging device Dolphin-View (Dolphin-View, Kurabo, Japan). The DNA sample amplified by PCR was purified using the ethanol precipitation method. The sequence was outsourced to the Hokkaido System Science Co., Ltd. Samples were sequenced using the PRISM3130 Genetic Analyzer (Applied Biosystems), and sequence data were analyzed with GENETYX software.

Statistical Analysis

Statistical analysis of *H. pylori* protein expression level measurement was performed using *t*-test. The *P*-value of <0.05 was considered statistically significant.

Results

H. pylori Growth Inhibition Test with Disulfiram

Whether disulfiram inhibits the growth of *H. pylori* was examined using a total of 12 strains. All strains underwent complete growth inhibition in the media with disulfiram concentrations of 10 μ g/mL and 5 μ g/mL. In the drug-sensitive (Figure 1A) and clarithromycin-resistant strains (Figure 1C), two strains were suppressed in growth in a medium with a disulfiram concentration of 1 μ g/mL, and one strain grew from the third day of culture. In the metronidazole-resistant strain (Figure 1D), two strains were suppressed in growth in a medium with a disulfiram concentration of 1 μ g/mL, and one strain grew from the 4th day of culture. Among the amoxicillin-resistant strains (Figure 1B), one strain underwent

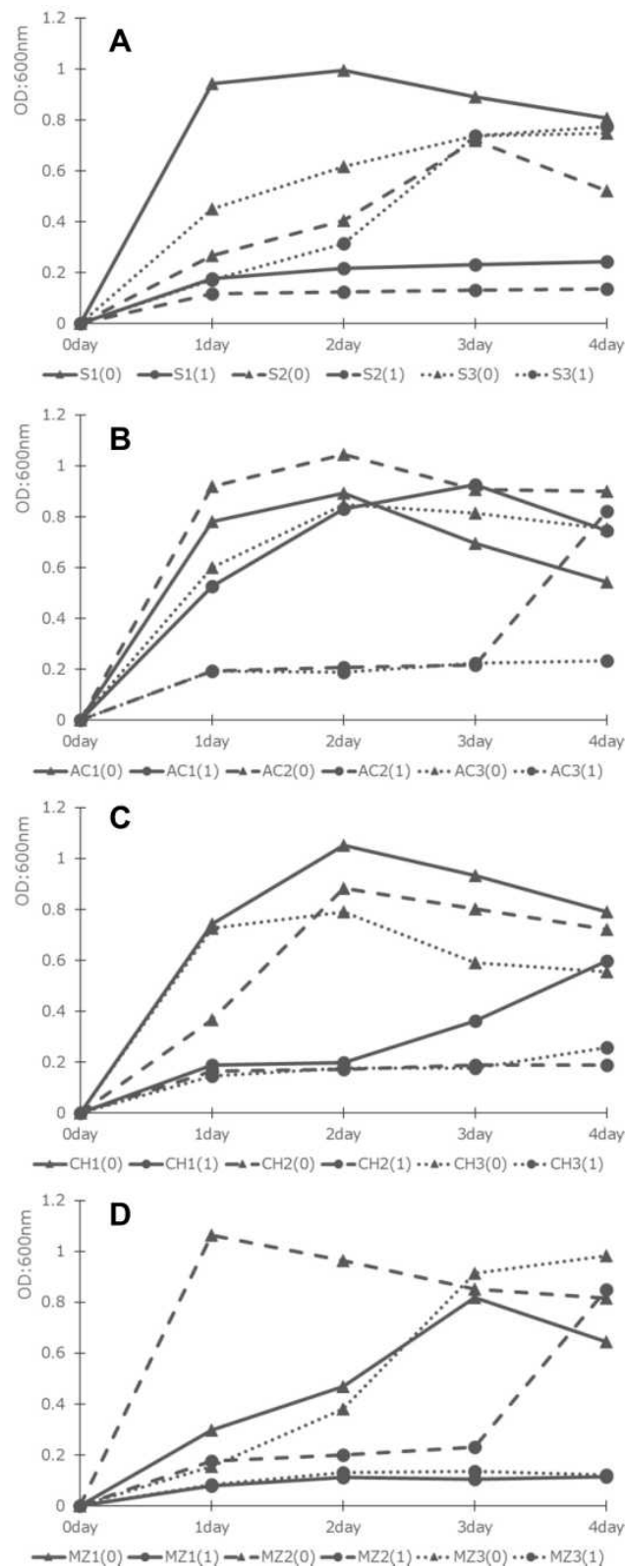


Figure 1 Inhibition of *H. pylori* growth at a disulfiram concentration of 1 µg/mL. **(A)** Drug-sensitive strain, **(B)** amoxicillin-resistant strain, **(C)** clarithromycin-resistant strain, and **(D)** metronidazole-resistant strain. The vertical axis represents the absorbance at OD₆₀₀ nm, and the horizontal axis represents the number of days of the culture. Numbers in parentheses indicate the disulfiram concentration (µg/mL).
Abbreviations: S, drug-sensitive strain; AC, amoxicillin-resistant strain; CH, clarithromycin-resistant strain; MZ, metronidazole-resistant strain.

growth inhibition in a medium with a disulfiram concentration of 1 $\mu\text{g}/\text{mL}$, and one strain grew from the 4th day of culture, and another one strain grew from the 1st day of culture. No correlation was found between susceptibility/resistance to current disinfectants and disulfiram resistance. After collecting the bacterial solution after shaking the culture and observing the morphological changes of the bacteria using the Gram stain, the spiral shape was lost and the bacteria changed to a short rod-like (Figure 2A and B). In addition, when applied to BHI agar medium with 7% horse blood and cultured at 37°C for 7 days, the amount of colonies was smaller than the usual growth (Figure 2C and D).

H. pylori Pathogenic Protein Expression Level Measurement

Bacterial proteins were compared with a disulfiram concentration of 0 $\mu\text{g}/\text{mL}$ and 1 $\mu\text{g}/\text{mL}$ using one from each of the drug-sensitive, amoxicillin-resistant, clarithromycin-resistant, and metronidazole-resistant strain with growth confirmed in the growth inhibition test. The increase/

decrease in urease, VacA, and CagA protein expressions levels in each bacterium was measured. In the drug-sensitive strain, protein expression levels of all three species decreased, and a significant difference was observed between urease and VacA (Figure 3A). Amoxicillin-resistant strains had significantly reduced protein expression levels in all three species (Figure 3B). In the clarithromycin-resistant strain, protein expression levels of all three species decreased, and a significant difference was observed in urease (Figure 3C). In the metronidazole-resistant strain, expression levels of urease and VacA decreased and that of CagA increased, and no significant difference was observed among the three species (Figure 3D).

Confirmation of Gene Mutation

For all 12 *H. pylori* strains used, the sequence of 16SrDNA, a ribosomal gene involved in protein production, was analyzed, and the presence or absence of gene mutation was examined. Table 3 shows the number of base substitutions and the number of deletions/insertions in the 16SrDNA

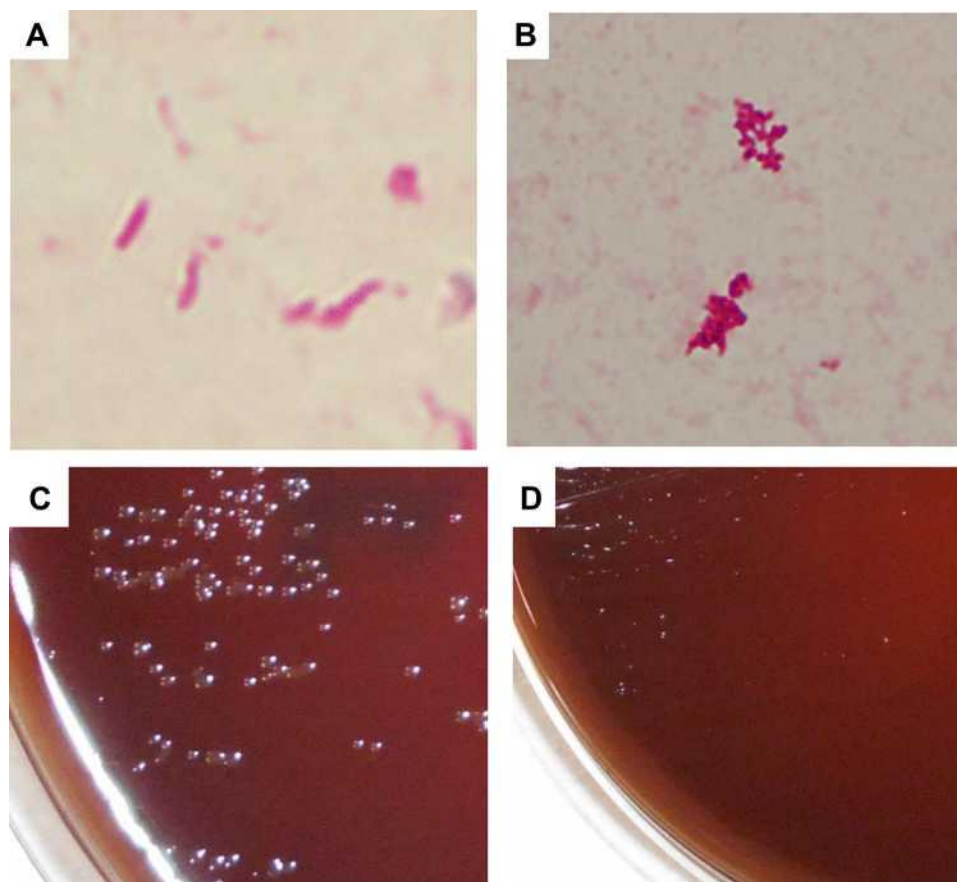


Figure 2 Bacterial morphology and colonies. (A) Morphology of *H. pylori* growth in BHI broth without disulfiram, (B) morphology of *H. pylori* with 1 μg of disulfiram. After cultivation in broth, bacteria were inoculated on BHI agar. (C) Colony of *H. pylori* transferred from BHI broth without disulfiram, (D) colony of *H. pylori* transferred from BHI broth with 1 μg of disulfiram.

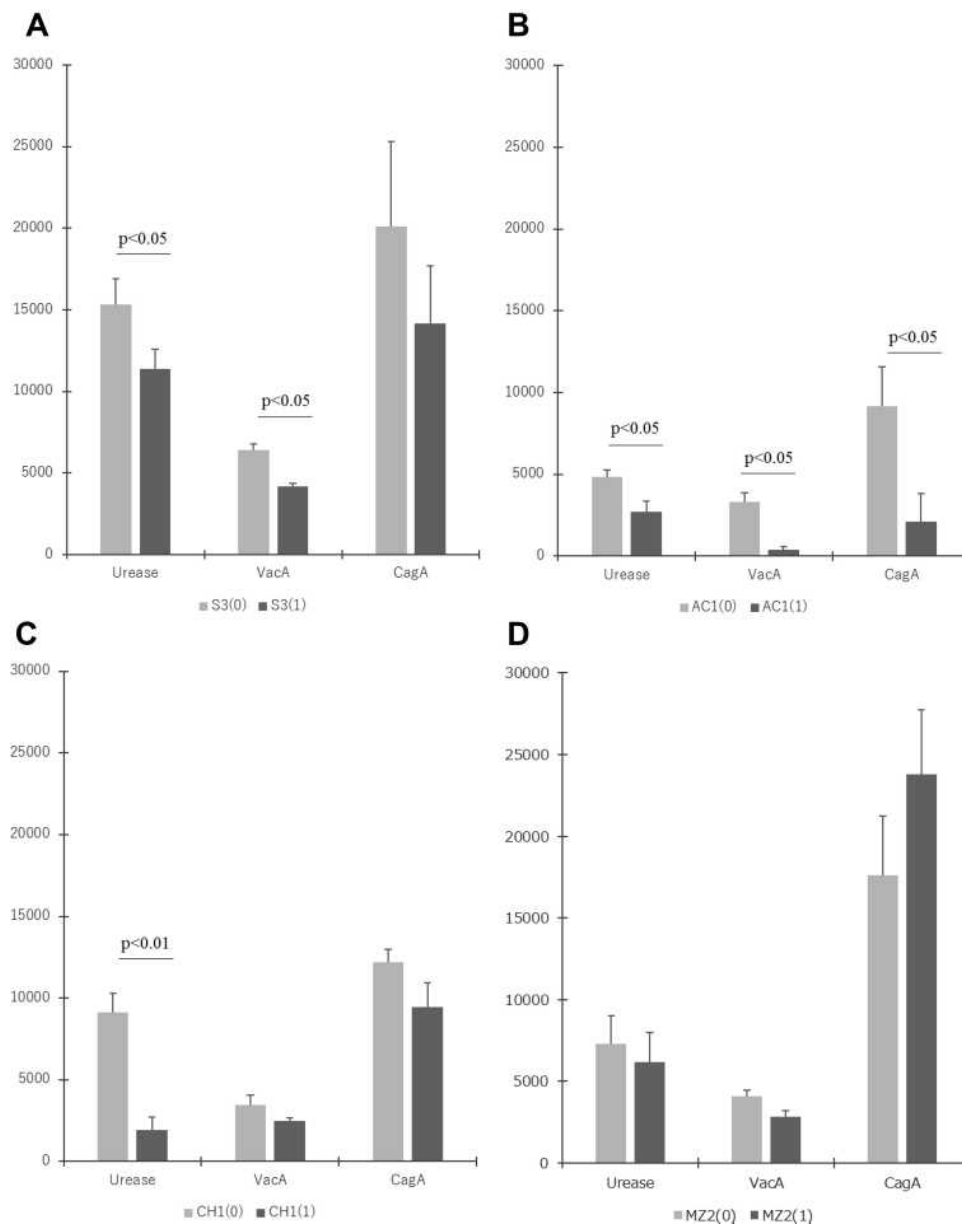


Figure 3 *H. pylori* pathogenic protein expression level. (A) Drug-sensitive strain, (B) amoxicillin-resistant strain, (C) clarithromycin-resistant strain, and (D) metronidazole-resistant strain. The vertical axis represents the protein expression level.

sequence. Of these, susceptible strain 3 (S3), amoxicillin-resistant strain 1 (AC1), amoxicillin-resistant strain 2 (AC2), clarithromycin-resistant strain 1 (CH1), and metronidazole-resistant strain 2 (MZ2) were resistant to disulfiram in the growth suppression test. Strains resistant to disulfiram tended to have fewer base deletions/insertions.

Discussion

Eradication therapy using amoxicillin and clarithromycin is performed for *H. pylori* infection, a risk factor for the occurrence of gastrointestinal diseases such as gastric

cancer. However, in recent years, a decrease in the eradication success rate due to an increase in drug-resistant strains has become a problem, and further increase in drug-resistant strains and the emergence of resistant strains to new antibiotics should be prevented.¹³

Disulfiram is an oral prescription drug mainly used as a therapeutic drug for alcohol dependence and is usually taken orally in 1–3 divided doses of 100–500 mg daily, and the maintenance dose is 100–200 mg.⁸ In this study, disulfiram suppressed the growth of 11 among 12 strains at a concentration of 1 µg/mL at 2 days of *H. pylori* culture

Table 3 16S rDNA Sequence

Strain Name	Number of Mutation	Number of Deletions/Insertions
S1	6	5
S2	1	2
S3	3	1
AC1	10	4
AC2	11	4
AC3	24	5
CH1	5	7
CH2	3	0
CH3	29	13
MZ1	8	10
MZ2	1	2
MZ3	6	8

and 7 at 4 days of culture. In addition, *H. pylori* acted on disulfiram showed poor growth of colonies in 7% horse blood BHI agar medium, and the cells lost their spiral shape and became short rod-like. Based on these facts, disulfiram is considered to exhibit an antibacterial effect against *H. pylori* at a low concentration regardless of the sensitivity and resistance to the current *H. pylori* eradication drug.

Urease, VacA, and CagA are important proteins with pathogenic effect in the stomach. *H. pylori*, which neutralized gastric acid with urease, secretes VacA, a cell-vacuolating toxin, destroys host cells, and secretes CagA protein to promote carcinogenesis of host cells.¹⁴ In this study, the expression levels of urease, VacA, and CagA proteins decreased in *H. pylori* infection treated with disulfiram. Based on the results of the growth inhibition test, disulfiram is considered to exhibit a concentration-dependent bactericidal action by inhibiting protein synthesis of *H. pylori*.

16SrDNA is a ribosomal gene involved in protein production, and this sequence of approximately 1500 bases is used for bacterial phylogeny. The 16SrDNA sequence is abundantly registered in public databases and is considered suitable for examining sequences specific to a particular bacterial species/group.^{15,16} When 16SrDNA mutations were confirmed in all 12 *H. pylori* strains used in this study, strains showing disulfiram resistance tended to possess fewer base deletions/insertions but were susceptible/resistant to disulfiram. No specific mutations due to differences were detected. Most of these deletions/insertions were found in domain 2 (V4 region) of 16SrDNA, and we cannot find that this fact is important or associate with protein production. For clearer features, the number

of samples should be increased, and further study conducted.

Conclusion

Although the genetic characteristics on the effectiveness of disulfiram against *H. pylori* can be further investigated, it exhibited a bactericidal effect on *H. pylori* at a lower concentration than when used as a supplemental drug. Therefore, disulfiram could be used as an adjunct to the current *H. pylori* eradication drug.

Abbreviations

AC, amoxicillin; CH, clarithromycin; MZ, metronidazole; BHI, brain heart infusion; MIC, minimum inhibitory concentration; PCR, polymerase chain reaction.

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Disclosure

The authors report no conflicts of interest in this work.

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