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(RESEARCH ARTICLE)

AcrB is essential for bile acid tolerance in *Helicobacter pylori*

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Abstract

Resistance to bile acids is a necessary ability for bacteria that live in the gastrointestinal tract, such as *Helicobacter pylori*. The role of the bacterial drug efflux system is important for bile acid tolerance. Using the *H. pylori* JP26 strain and the JP26AcrB strain deficient in *AcrB* the relationship between the drug efflux system AcrB and the bile acid tolerance of *H. pylori* was investigated. Bacterial susceptibility to bile acids was measured by the agar dilution method. To examine the ability to efflux bile acids, the intracellular accumulation of radiolabeled chenodeoxycholic acid was measured with a liquid scintillation counter. To investigate the effect of bile acid on the cell adhesion ability of bacteria, bacteria were attached to AGS cells supplemented with chenodeoxycholic acid, and the number of bacteria was counted. Bile acid resistance was significantly lower in the *AcrB* mutant than in the wild-type strain. The *AcrB* mutant strains showed a significant increase in the accumulation of ^{14}C - labeled chenodeoxycholic acid in the bacteria. In terms of attachment to AGS cells, the *AcrB* mutant was significantly lower than the wild-type strain. Furthermore, the *AcrB* mutant with chenodeoxycholic acid could not be identified. Our results reveal that *H. pylori* AcrB plays an important role in bile acid resistance. We also suggest that drugs targeting the AcrB efflux system may be a new treatment for *H. pylori*.

Keywords: *Helicobacter pylori*; AcrB; Bile acid; Susceptibility; Efflux; AGS cell

1. Introduction

Gastric pathogen, *Helicobacter pylori* causes gastric ulcer and gastric cancer [1, 2, 3]. In many developing countries it infects about 80% of the population and in developed countries it infects about 30% [2,3]. *H. pylori* plays an important role in the pathogenesis of chronic gastritis, peptic ulcer diseases, gastric carcinomas, and gastric marginal zone B-cell lymphomas of mucosa-associated lymphoid tissue type [4, 5, 6, 7].

As the standard treatment for *H. pylori* is the use of antibacterial drugs, bile reflux gastritis occurs in the absence of *H. pylori* [8]. Inhibition of *H. pylori* growth by bile also suggests that it should be difficult for *H. pylori* to colonize the duodenum and cause duodenal ulcer [9]. These finding suggested that bile salts might be useful in the elimination of *H. pylori*. Although it has been shown that bile acids possess antibacterial activity against *H. pylori,* few reports on their precise mechanism of activity have been shown [8, 9, 10].

Gastrointestinal tract related bacteria such as *H. pylori* must tolerate high levels of bile salts, powerful detergents that disrupt biological membranes. The outer membrane barrier of gram-negative bacteria plays an important role in this resistance, but ultimately it can only retard the influx of bile salts [11]. For example, *Escherichia coli* possessed an energy-dependent efflux mechanism for these compounds [12]. Growth inhibition of *E. coli* by bile salts and

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accumulation levels of chenodeoxycholate increased when mutations inactivating the *acrAB* gene clusters were introduced. The AcrAB system especially appeared to play a significant role in bile acid efflux [13, 14].

In the only reported study of the relationship between the AcrB system of *H. pylori* and bile acids, the *hefF* and *hefI* of the AcrB system appear to be insignificant, indicating that *hefC* (*AcrB*) plays an important role in bile acid resistance [15]. However, no further studies have been conducted on the relationship between the *H. pylori* efflux system AcrB and bile acids. Therefore, we investigated whether this AcrB efflux systems of *H. pylori* affect bile acid tolerance.

2. Material and methods

2.1. Bacterial strains, culture condition

Strains and plasmids used in this study are listed in Table 1 [16, 17]. *E. coli* was routinely grown at 37°C in Luria-Bertani broth or agar supplemented with ampicillin.

Table 1 Bacterial strains and plasmids used in this study

Strain (alternate name) or plasmid	Genotype or characteristics	Reference or source
JP26	*H. pylori* wild -type	[16]
JP26AcrB	JP26 / HP607: *aphA*	This study
pGEMT Easy	ColE1, Ampr, PCR cloning vector	Promega
pUC4K	ColE1, Amp^r, *aphA*	Pharmacia
pGEMT AcrBKm	pGEMT Easy / HP607: *aphA*	[17]

(100 μg/ml), and kanamycin (Km) (25 μg/ml), when appropriate. *H. pylori* strains were grown on Trypticase soy agar (TSA) with 5% sheep blood (Becton Dickinson and Company, MD, USA) or Brucella serum (Becton Dickinson and Company) agar with 10% newborn calf serum (FCS; Gibco BRL, MD, USA) plates at 37°C in 5%CO_2 atmosphere. Antibiotic-resistant *H. pylori* transformants were selected with Km (25 μg/ml). Bile acid was purchased from Sigma Aldrich co. Cholic acid, deoxycholic acid, and chenodeoxycholic acid were purchased from Fuji film wako pure chemical Co. (Osaka, Japan).

2.2. Construction of plasmids and *H. pylori* mutant strains

H. pylori chromosomal DNA was prepared from cells of each strain after 48 h of growth on two agar plates as described previously [18, 19, 20]. Plasmid DNA was prepared from *H. pylori* after 48 h of growth or from *E. coli* after overnight cultures, using a midi-prep protocol (Qiagen N.V., Venlo, CA, USA) according to the manufacturer's instructions. The HP607 ORF of strain *H. pylori* JP26 were amplified by PCR using primers (HP607sense, and HP607antisense), respectively (Table 2) [16, 17]. The product was ligated into pGEM-T Easy (Promega Co, WI, USA) and transformed into *E. coli* DH5α. A unique EcoRI site was created by inverse PCR (HP607inv1, HP607inv2).

Table 2 Oligonucleotide primers used in this study

Primer	Sequence
HP0607sense	cggtggtggttactacgact
HP0607antisense	gagcaaaatcaagcctatca
HP0607inv1	ggaattcacttcagcatgtttttcaatcg
HP0607inv2	ggaattcaccaccttgcaagtgggtta

Plasmid pUC4K was digested with EcoRI (Takara Bio Inc., Ohtsu, Japan), after which the Km resistance (Kan^r; *aphA*) cassette was isolated by agarose gel electrophoresis and ligated into the inverse PCR product to disrupt the HP607 ORF, creating pGEMTAcrBkm. *H. pylori* JP26 was transformed to Kan^r with pGEMTAcrB km to create JP26AcrB by

electroporation as described elsewhere [18, 19], resulting in strain JP26AcrB (Table 1). Chromosomal DNAs isolated from *H. pylori* mutants were confirmed by PCR. PCR oligonucleotide primers specific for HP607 or *aphA* were used, and the sizes of the PCR products were evaluated by agarose gel electrophoresis (Table 2).

2.3. Bile acid susceptibility testing

The bile acid derivatives susceptibilities of the wild-type and *AcrB* mutant *H. pylori* strains were determined by the agar dilution method [17]. The susceptibility of bile acid derivatives were defined as the lowest concentration that inhibited the visible growth of isolates completely by the agar dilution method on Mueller-Hinton agar (Becton Dickinson and Company) plates supplemented with 5% sheep blood (Becton Dickinson and Company) (M-H agar). Briefly, all isolates were incubated for 4 days on BB agar. After this incubation, inocula were prepared by suspending growth from the BB agar plates with antimicrobial agents in saline to achieve a suspension equivalent to a 2.0 McFarland standard. Final inocula of 10^6 CFU/spot were applied to M-H agar or BB. All plates were incubated for 3 days at 35°C in a microaerophilic atmosphere, and the number of CFU was counted. In the liquid culture study, aliquots from each culture were applied to BB agar after 3 days of incubation of *H. pylori* at 37°C under microaerophilic conditions. After 4 days of incubation, the number of CFU was counted.

2.4. Determination of radiolabeled chenodeoxycholic acid uptake

To investigate the efflux ability of bile acid, intracellular accumulation of radiolabeled chenodeoxycholic acid in the *H. pylori* JP26 strain, and *AcrB*-mutant strains was measured by a liquid scintillation counter. At 20 h before the end of the *H. pylori* culture, carboxyl-^{14}C-chenodeoxycholic acid [ARC0856] [50mCi/mmol] [Muromachi Kikai Co., Ltd. Tokyo. Japan] was added to the medium in the wells. To investigate the efflux ability of chenodeoxycholic acid, intracellular accumulation of ^{14}C in each strain was measured at every 30 sec by a liquid scintillation counter after incubation with carboxyl-^{14}C-chenodeoxycholic acid. When the culture was finished, the cells were adsorbed on 0.45 μm membrane filters (Advantech Japan, Tokyo, Japan), washed with distilled water, and then dried. The filters were transferred to vials filled with liquid scintillator cocktail, and the radioactivity was measured with a liquid scintillation counter (LSC-6100, Hitachi Aloka Medical, Tokyo, Japan).

2.5. AGS cell adhesion experiment

H. pylori strains were grown on Trypticase soy agar (TSA) with 5% sheep blood in 5% CO_2 atmosphere. The bacteria were harvested and resuspended in RPMI 1640 medium (Fuji film wako). AGS cells that stocked in department of gastroenterology, Nagoya University, were grown to confluence in 12 well flat bottom microplate (AGC Inc., Tokyo, Japan). Each AGS monolayer was washed twice with RPMI 1640 medium. *H. pylori* was added at a multiplicity of infection of more than 1,000:1 and incubated at 37°C in 5% CO_2 for a total of 4 h. An identical amount of *H. pylori* was added to a microplate without AGS cells and incubated in the same way as the *H. pylori*-AGS cell coculture. A noninfected AGS cells served as a negative control. After 1 h of coincubation, the *H. pylori*-AGS cell coculture was washed twice with RPMI 1640 medium to remove unattached *H. pylori cells* and debris, which eliminated unattached organisms from the analysis. The RPMI 1640 wash medium was prewarmed at 37°C to avoid stressing either *H. pylori* or the AGS cells. One hour was sufficient time for tight attachment of *H. pylori* to AGS cells, as confirmed by microscopy. After washing, incubation was continued for an additional 3 h. At the end of the incubation period, the *H. pylori*-AGS cell coculture was washed two times with phosphate-buffered saline (pH 7.4, 37°C) to remove detached *H. pylori* cells. All the control flasks were treated in the same way. After May-Grunwald-Giemsa staining, the number of bacterial cells were measured at 6 different high-powered field (HPF) of the specimen under × 1000 magnification, and their average was used as the result from one individual [21].

2.6. Statistical analysis

All the experiments were repeated at least three times, and the results were expressed as the mean values ± standard deviations and compared using a paired *t* test. A *p* value of less than 0.05 was considered a significant difference.

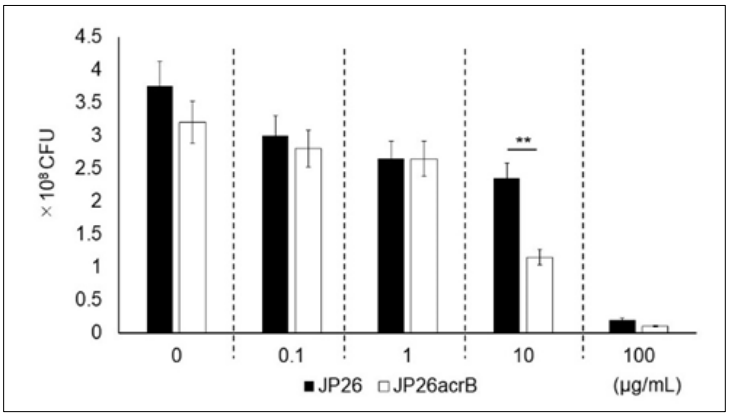
3. Results

3.1. Construction of -*AcrB* mutants and determination of bile acid derivatives susceptibility

H. pylori AcrB mutant was created from *H. pylori* JP26 strain. Bile acids significantly inhibited bacterial growth of *AcrB* mutant strains more than wild-type strains at a concentration of 10 μg/mL ($p < 0.01$), and both wild-type and *AcrB* mutant strains were markedly inhibited at concentrations of 100 μg/mL or higher, with no significant difference (Figure 1A).

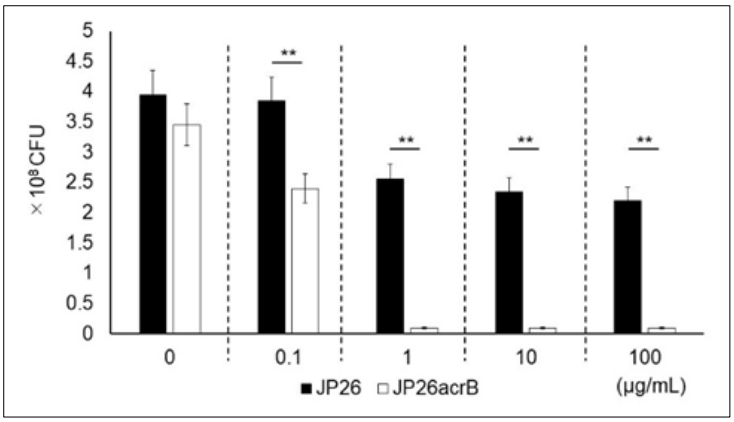
Cholic acid significantly inhibited the bacterial growth of *AcrB* mutant strains more than wild-type strains at a concentration of 0.1 μg/mL ($p < 0.01$), and cholic acid at a concentration of 100 μg/mL or higher markedly inhibited both wild-type and *AcrB* mutant strains with no significant difference (Figure 1B).

Deoxycholic acid also significantly inhibited the bacterial growth of *AcrB* mutant strains more than wild-type strains at a concentration of 0.1 μg/mL ($p < 0.01$), and deoxycholic acid at a concentration of 100 μg/mL or higher markedly inhibited both wild-type and *AcrB* mutant strains with no significant difference (Figure 1C).



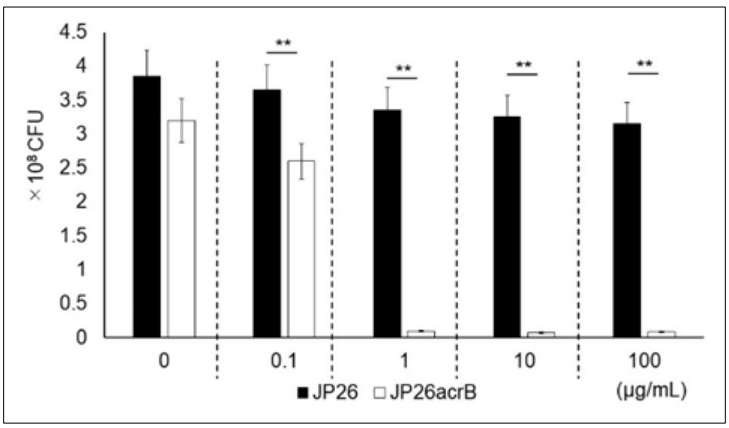
**: $p < 0.01$

Figure 1A Comparison of bile acid susceptibility among *H. pylori*



**: $p < 0.01$

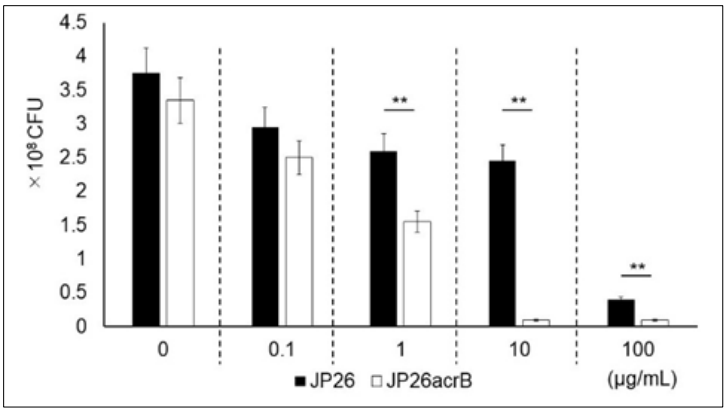
Figure 1B Comparison of cholic acid susceptibility among *H. pylori*



**: $p < 0.01$

Figure 1C Comparison of deoxycholic acid susceptibility among *H. pylori*

Chenodeoxycholic acid at a concentration of 1 μg/mL significantly inhibited the bacterial growth of *AcrB* mutant strains more than wild-type strains ($p < 0.01$), and at a concentration of 10 μg/mL or higher, both wild-type and *AcrB* mutant strains were significantly inhibited, with no significant difference (Figure 1D).

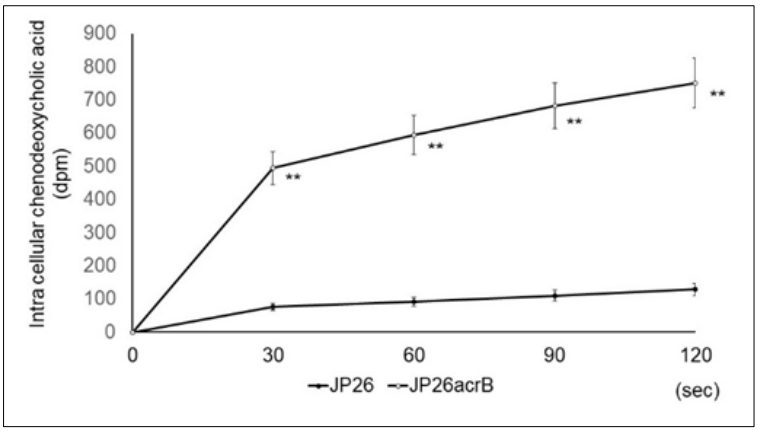


**: $p < 0.01$

Figure 1D Comparison of chenodeoxycholic acid susceptibility among *H. pylor*

3.2. Ability of AcrB to efflux chenodeoxycholic acid from bacterial cells

Next, we examined the difference in macrolide efflux capacity between the wild strain and the *AcrB* mutant strains. Chenodeoxycholic acid labeled with ^{14}C was added to *H. pylori* and incubated, and ^{14}C in the bacteria was measured by liquid scintillation counter. Throughout the entire course of the study, a marked increase in ^{14}C levels was observed in *AcrB* mutant strains compared to wild-type strains ($p< 0.01$) (Figure 2). From these results, we suggested that the intracellular chenodeoxycholic acid concentration significantly increased in the *AcrB*-mutant strains than the *H. pylori* wild-type strains.

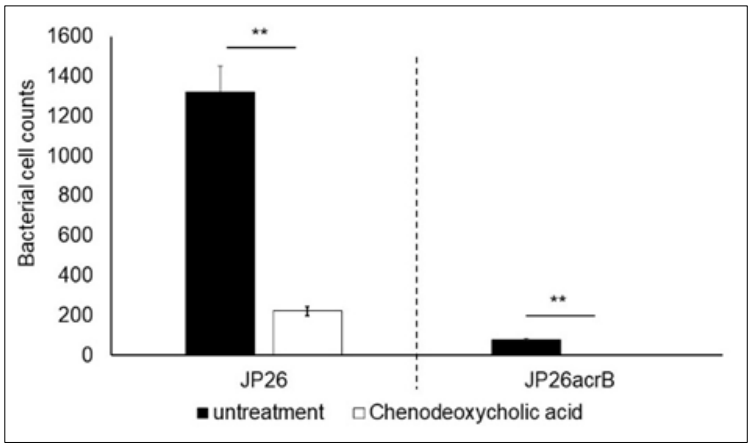


** : $p < 0.01$

Figure 2 Comparison of chenodeoxycholic acid efflux among *H. pylori*

3.3. AGS cell adhesion experiment

Finally, we examined the relationship between AGS cell adhesion and drug efflux factors such as AcrB. The number of *H. pylori* colonies in the chenodeoxycholic acid-treated group was significantly depressed relative to that in the control group ($p< 0.01$). The number of *AcrB* mutant *H. pylori* colonies were also significantly depressed relative to wild-type group ($p< 0.01$). Furthermore, the *AcrB* mutant strains to which chenodeoxycholic acid was added could not be confirmed to have bacteria adhering to the AGS cells ($p < 0.01$) (Figure 3).



** : $p < 0.01$

Figure 3 Comparison of AGS cell adhesion by chenodeoxycholic acid between *H. pylori*

4. Discussion

In this study, we showed that AcrB was involved in the bile acid resistance of *H. pylori*. Our result demonstrated that inactivation of AcrB increased the susceptibility of bile acid derivatives. AcrB was also found to be closely related to the efflux of bile acid derivatives from the bacterial cell and AGS cell attachment.

Some researcher investigated whether the bile acids chenodeoxycholic acid affected the growth or adherence of *H. pylori in vitro*. Twenty-seven strains' growth were inhibited by 0.1% chenodeoxycholic acid. Chenodeoxycholic acid was a more effective inhibitor of adherence in that the number inhibited [9]. This result is the basis for our use of chenodeoxycholic acid in the study of efflux and adhesion. Other researchers investigated the relationship between *H. pylori* strain (duodenal ulcer vs gastritis), type of bile acid conjugate, and inhibition of *H. pylori* growth. Synthetic human bile with or without lecithin inhibited *H. pylori* growth in a dose-dependent manner. There was no difference in inhibition between *H. pylori* gastritis and duodenal ulcer isolates [10]. Previous report showed that the morphology of *H. pylori* changed from its primary rod-like shape to a spherical shape with blebs on the cell surface, and was further degraded to an irregularly condensed mass, following an increase in bile acid. Moreover, this morphologic change in *H. pylori* was different from the change to a spherical shape caused by amoxicillin [22].

There are several reports investigating the relationship between bile acids and AcrB using the gut microbiota Salmonella. *Salmonella serotype typhimurium* transpositional mutant altered in resistance to biliary salts. This chromosomal DNA segment flanking transposon insertion was like the AcrB gene of *E. coli*, a gene encoding a drug efflux pump of the Acr family. This mutant exhibited a reduced capacity to colonize the intestinal tract. However, after passages in mice, the mutant strain lost the sensitive phenotype [23].

The decrease in resistance levels to bile salts was 64-fold higher for the AcrB mutants than to those of the wild-type *S. Typhimurium s*trains. But the colonization levels of the AcrB mutants were not significantly different from those of the wild-type strains [24]. Our adherence studies show that the bacterial count of *AcrB* mutants is low and further study is needed.

As the mechanism responsible for bile susceptibility in three deoxycholate-sensitive strains of *Salmonella enterica subspecies enterica serovar Pullorum* was investigated, the expression of *AcrB* mRNA was 10-fold lower in the deoxycholate-sensitive strains than in a deoxycholate-resistant strain. These results suggested that low expression of *AcrB* was strongly correlated with bile susceptibility in the deoxycholate-sensitive strains [25].

Previous study showed that the absence of the AcrB system significantly affected the colony morphology and outer membrane structure in a normal situation, compared with those of the wild-type *Cronobacter malonaticus*. The deletion of them caused the decline in resistance to bile salt stress, inhibition of growth, and observable reduction in relative growth rate and motility. Moreover, the bacterial stress response promoted the biofilm formation ability of the mutant strain [26].

5. Conclusion

Our results reveal that *H. pylori* AcrB plays an important role in bile acid resistance. Further studies on the AcrB of *H. pylori* may provide more detailed information on the mechanism of the bacterial efflux systems. This result may trigger the development of new therapeutic agents that target the AcrB system to enhance antibacterial effects other than direct antibacterial treatment.

Compliance with ethical standards

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Disclosure of conflict of interest

All authors declare no conflict of interest of regarding the publication of this paper.

References

[1] Dunn BE, Cohen H, Blaser MJ. Helicobacter pylori. Clin. Microbiol. Rev. 1997; 10: 720-741.

[2] Sung JJY, Chung S, Ling TKW, Yung MY, Leung VKS, Ng EKW, Li MKK, Cheng AFB, Li AKC. Antibacterial treatment of gastric ulcers associated with Helicobacter pylori. N Engl J Med. 1995; 332: 139-142.

[3] Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, Sibley RK. Helicobacter pylori infection and the risk of gastric carcinoma. N Engl J Med. 1991; 325: 1127-1131.

[4] Hiyama T, Tanaka S, Masuda H, Shima H, Kose K, Tuncel H, Ito M, Kitadai Y, Sumii M, Uemura N, Yoshihara M, Shimamoto F, Haruma K, Chayama K. Prevalence of Helicobacter pylori resistance to clarithromycin and metronidazole determined by 23S ribosomal RNA and rdxA gene analyses in Hiroshima, Japan. J Gastroenterol Hepatol. 2003; 18: 1202-1207.

[5] Masuda H, Hiyama T, Yoshihara M, Tanaka S, Haruma K, Chayama K. Characteristics and trends of clarithromycin-resistant Helicobacter pylori isolates in Japan over a decade. Pathobiol. 2004; 71: 159-163.

[6] Miyata E, Kudo T, Ikuse T, Tokita K, Arai N, Oka I, Kyodo R, Sato M, Hosoi K, Jimbo K, Aoyagi Y, Ohtsuka Y, Shimizu T. Eradication therapy for Helicobacter pylori infection based on the antimicrobial susceptibility test in children: A single-center study over 12 years. Helicobacter. 2021; 26: e12764.

[7] O'Connor A, Furuta T, Gisbert JP, O'Morain C. Review - Treatment of Helicobacter pylori infection 2020. Helicobacter. 2020; 25 Suppl 1: e12743.

[8] Huang H, Tian J, Xu X, Liang Q, Huang X, Lu J, Yao Y. A study on the roles of Helicobacter pylori in bile reflux gastritis and gastric cancer. J BUON. 2018; 23: 659-664.

[9] Mathai E, Arora A, Cafferkey M, Keane CT, O'Morain C. The effect of bile acids on the growth and adherence of Helicobacter pylori. Aliment Pharmacol Ther. 1991; 5: 653-658.

[10] Han SW, Evans DG, el-Zaatari FA, Go MF, Graham DY. The interaction of pH, bile, and Helicobacter pylori may explain duodenal ulcer. Am J Gastroenterol. 1996; 91: 1135-1137.

[11] Drew D, Klepsch MM, Newstead S, Flaig R, De Gier JW, Iwata S, Beis K. The structure of the efflux pump AcrB in complex with bile acid. Mol Membr Biol. 2008; 25: 677-682.

[12] Paul S, Alegre KO, Holdsworth SR, Rice M, Brown JA, McVeigh P, Kelly SM, Law CJ. A single-component multidrug transporter of the major facilitator superfamily is part of a network that protects Escherichia coli from bile salt stress. Mol Microbiol. 2014; 92: 872-884.

[13] Ma D, Cook DN, Alberti M, Pon NG, Nikaido H, Hearst JE. Genes acr A and acr B encode a stress-induced efflux system of Escherichia coli. Mol Microbiol. 1995; 16: 45-55.

[14] Thanassi DG, Cheng LW, Nikaido H. Active efflux of bile salts by Escherichia coli. J Bacteriol. 1997; 179: 2512-2518.

[15] Trainor EA, Horton KE, Savage PB, Testerman TL, McGee DJ. Role of the HefC efflux pump in Helicobacter pylori cholesterol-dependent resistance to ceragenins and bile salts. Infect Immun. 2011; 79: 88-97.

[16] Minami M, Hashikawa SN, Ando T, Kobayashi H, Goto H, Ohta M. Involvement of CO2 generated by urease in multiplication of Helicobacter pylori. GSC Advanced Research and Reviews. 2021; 07(03): 045–053.

[17] Minami M, Hashikawa SN, Ando T, Goto H, Ohta M. AcrB-TolC efflux system is essential for macrolide resistance in Helicobacter pylori. GSC Advanced Research and Reviews. 2021; 09(03): 056–062.

[18] Ando T, Israel DA, Kusugami K, Blaser MJ. HP0333, a member of the dprA family, is involved in natural transformation in Helicobacter pylori. J Bacteriol. 1999; 181: 5572–5580.

[19] Minami M, Ando T, Hashikawa SN, Torii K, Hasegawa T, Israel DA, Ina K, Kusugami K, Goto H, Ohta M. Effect of glycine on Helicobacter pylori in vitro. Antimicrob Agents Chemother. 2004; 48: 3782-3788.

[20] CLSI. Performance standards for antimicrobial susceptibility testing: M100, 30th ed, PA: Clinical and Laboratory Standard Institute; 2020.

[21] Kim N, Marcus EA, Wen Y, Weeks DL, Scott DR, Jung HC, Song IS, Sachs G. Genes of Helicobacter pylori regulated by attachment to AGS cells. Infect Immun. 2004; 72: 2358-2368.

[22] Itoh M, Wada K, Tan S, Kitano Y, Kai J, Makino I. Antibacterial action of bile acids against Helicobacter pylori and changes in its ultrastructural morphology: effect of unconjugated dihydroxy bile acid. J Gastroenterol. 1999; 34: 571-576.

[23] Lacroix FJ, Cloeckaert A, Grépinet O, Pinault C, Popoff MY, Waxin H, Pardon P. Salmonella typhimurium AcrB-like gene: identification and role in resistance to biliary salts and detergents and in murine infection. FEMS Microbiol Lett. 1996; 135: 161-167.

[24] Baucheron S, Mouline C, Praud K, Chaslus-Dancla E, Cloeckaert A. TolC but not AcrB is essential for multidrug-resistant Salmonella enterica serotype Typhimurium colonization of chicks. J Antimicrob Chemother. 2005; 55: 707-712.

[25] Usui M, Asai T, Sato S. Low expression of AcrB in the deoxycholate-sensitive strains of Salmonella enterica subspecies enterica serovar Pullorum. Microbiol Immunol. 2011; 55(5): 366-368.

[26] Wang X, Wang Y, Ling N, Shen Y, Zhang D, Liu D, Ou D, Wu Q, Ye Y. Effects of tolC on tolerance to bile salts and biofilm formation in Cronobacter malonaticus. J Dairy Sci. 2021; 104: 9521-9531.