

## *Helicobacter pylori* May Survive Ampicillin Treatment in the Remnant Stomach

YOSHIO HOSAKA<sup>a,b</sup>, RYOICHI OKAMOTO<sup>a</sup>, KAZUHIKO IRINODA<sup>a</sup>, SATORU KAIEDA<sup>a</sup>, WASABURO KOIZUMI<sup>b</sup>,  
KATUNORI SAIGENJI<sup>b</sup> and MATSUHISA INOUE<sup>\*a</sup>

<sup>a</sup>Department of Microbiology, Kitasato University School of Medicine,

<sup>b</sup>The First Department of Internal Medicine, Kitasato University School of Medicine,  
1-15-1 Kitasato, Sagami-hara-shi, Kanagawa 228-8555, Japan

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*Helicobacter pylori* (*H. pylori*) is a Gram-negative curved rod-like or spiral bacterium that chronically infects the human gastric mucosa, and is a major risk factor for gastritis, gastric and duodenal ulcer and adenocarcinoma of the stomach. After partial gastrectomy, some patients may have persistent *H. pylori* infection for five years or more. In this study, we detected three bacteria, *i.e.*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Escherichia coli*, in the gastric juice of patients with a remnant stomach. Some of these bacteria produced  $\beta$ -lactamase. These findings are potentially important since such bacteria could provide *H. pylori* with the chance to acquire drug resistance and to transfer drug resistance genes. This could be one reason why *H. pylori* is difficult to eradicate in the remnant stomach.

*Helicobacter pylori* is a Gram-negative curved rod-like or spiral bacterium that chronically infects the gastric mucosa in humans<sup>1</sup>). It is a major risk factor for gastritis, gastric or duodenal ulcer<sup>2~4</sup>) and adenocarcinoma of the stomach<sup>5,6</sup>). Following partial gastrectomy, it has been reported that the rate of *H. pylori* infection decreases over time, but some patients still have *H. pylori* infection after five years or more<sup>7,8</sup>). In this study, we investigated the bacteria in the gastric juice of patients with a remnant stomach to determine whether any of these bacteria produced  $\beta$ -lactamase and resistance to  $\beta$ -lactam antibiotics of  $\beta$ -lactamase non-producing *H. pylori*.

### Materials and Methods

#### Bacterial Strains and Plasmids

The *Escherichia coli* KU2273 derived from K-12 strain and bearing plasmid RP4 as well as American Type Culture Collection (ATCC) *H. pylori* 43629 were used. Several *E. coli* (KU6269 and KU6270), *Enterobacter aerogenes* (KU6271, KU6272, and KU6273) and *Klebsiella pneumoniae* (KU6274, KU6275, and KU6276) strains were clinical isolates from the gastric juice of patients with a remnant stomach who were admitted to the

Gastroenterology ward of Kitasato University Hospital in 1999.

#### Culture Medium

Test strains for the sensitivity assays were incubated in Mueller Hinton broth and agar (MH, Nissui, Tokyo, Japan). L-broth containing 5 g of peptone tryptone (Difco Laboratories, MI, USA), 2.5 g of yeast extract (Difco Laboratories) and 2.5 g of sodium chloride (Wako, Pure Chemical, Industries, Tokyo, Japan) was used for the assay of  $\beta$ -lactamase activity and for mixed culture experiments.

*H. pylori* was grown for 4 days on trypto-soy agar (TSA; Eiken Chemical, Tokyo, Japan) supplemented with 5% sheep blood at 37°C in an incubator with an atmosphere of 10.5% CO<sub>2</sub>.

#### Test Drugs

Several antibacterial agents were tested in this study. The respective manufacturers kindly provided reference powders for the following drugs of known potency: ampicillin (AMP, Meiji Seika Kaisha, Tokyo, Japan), piperacillin (PIPC, Toyama Chemical, Tokyo, Japan), cephalothin (CET, Shionogi Seiyaku, Osaka, Japan), ceftazidime (CAZ, Glaxo SmithKline, Tokyo, Japan), imipenem (IPM, Banyu Seiyaku, Tokyo, Japan) and

\* Corresponding author: matsu@kitasato-u.ac.jp

nalidixic acid (NA, Sigma, Tokyo, Japan).

#### Determination of MICs

Drug resistance was assessed by determining the minimum inhibitory concentration (MIC) that inhibited the visible growth of bacteria using the agar dilution method. Briefly, after overnight incubation at 35°C in MH broth, medium containing bacteria was diluted in phosphate-buffered saline with gelatin to a density of  $5\sim 6 \times 10^6$  CFU/ml. Then 5- $\mu$ l aliquots were inoculated directly onto an MH agar plate containing a dilution of an antibiotic using a multiple-spot plating device (Sakumaseisakujyo, Tokyo). All plates (including control plates) were incubated at 35°C for 18 hours.

#### Preparation and Assay of $\beta$ -Lactamase

Test strains were incubated overnight in L-broth at 35°C for 18 hours. Then the bacteria were diluted 20-fold with 10 ml of fresh L-broth, and were incubated at 35°C with shaking. Microorganisms in the late logarithmic growth phase were collected by centrifugation at  $18,000 \times g$  for 10 minutes, washed with 50 mM phosphate buffer (pH 7.0), and suspended in 3 ml of the same buffer. This bacterial suspension was sonicated and then was centrifuged at  $18,000 \times g$  for 20 minutes at 4°C. The supernatant was used as the crude enzyme preparation<sup>9</sup>). Enzyme activity was determined by spectrophotometry (UV2000, Shimadzu Corp., Tokyo) at 30°C in 50 mM phosphate buffer (pH 7.0) using penicillin G as the substrate.

#### Detection of Enterobacteria in Gastric Juice from the Remnant Stomach

We examined gastric juice from 18 patients who had previously undergone distal gastrectomy and were received

upper gastrointestinal endoscopy in the Gastroenterology ward of Kitasato University Hospital in 1999. A 10- $\mu$ l aliquot of each sample was spread on DHL agar plates (Eiken Chemical, Tokyo, Japan) and BTB agar plates (Eiken Chemical) and was incubated at 35°C for 16 hours. We isolated and counted the number of colonies, and twice performed purification. After purification, these bacteria were detected using BIOTEST 1 (Eiken Chemical).

#### Mixed Culture of *E. coli* and *H. pylori*

*E. coli* were incubated overnight in L-broth at 35°C for 18 hours. Then these cells were diluted 20-fold with 10 ml of fresh L-broth and were incubated at 35°C with shaking for 3 hours. *H. pylori* harvested from the plates were suspended in 5 ml of TSB supplemented with 10% horse serum (contained about  $1 \times 10^{7\sim 8}$ ) and were cultured with shaking for 24 hours at 35°C in a gas mixture (N<sub>2</sub>, 85%; O<sub>2</sub>, 5%; and CO<sub>2</sub>, 10%).

After that, an equal volume (0.5 ml) of each bacterial strains was mixed and incubated for 6 hours at 37°C under an atmosphere of 10.5% CO<sub>2</sub>. After incubation, 100  $\mu$ l of the mixed culture fluid was spread onto agar containing both NA (25  $\mu$ g/ml) and AMP (0.5  $\mu$ g/ml) and then incubated for 3 days, after which it was checked for growth of *H. pylori*.

### Results and Discussion

We isolated one or more species of bacteria from the gastric juice obtained from 6 of 18 patients with a remnant stomach. These bacteria were *K. pneumoniae*, *E. aerogenes*, and *E. coli* (Table 1). In this study, we did not investigate the presence of *H. pylori* in any of these patients. Other

Table 1. Bacteria isolated from the gastric juice of patients with a remnant stomach.

Species	No. of patients	Viable cell count (CFU/ml)
<i>Klebsiella pneumoniae</i>	2	$1.0 \times 10^3$
<i>Enterobacter aerogenes</i>	3	$1.0 \times 10^{2-4}$
<i>Escherichia coli</i>	2	$2.2 \times 10^3$
None	12	-

One patient had both *Klebsiella pneumoniae* and *Escherichia coli*.

Table 2. MIC values (mg/ml) for different antibiotics and hydrolysis of penicillin G by microorganisms isolated from gastric juice in patients with a remnant stomach.

Bacterial species	MIC ( $\mu$ g/ml)			$\beta$ -lactamase activity (U/mg of protein)	** Inducible resistance
	AMP	PIPC	CET		
<i>Escherichia coli</i>					
KU6269	8	2	4	<0.01	NT
KU6270	8	2	4	<0.01	NT
<i>Enterobacter aerogenes</i>					
KU6271	1024	4	128	<0.01	+
KU6272	512	2	128	<0.01	+
KU6273	512	2	256	<0.01	+
<i>Klebsiella pneumoniae</i>					
KU6274	64	4	2	0.078	NT
KU6275	16	2	2	<0.01	NT
KU6276	16	2	2	<0.01	NT

\* Average of three separate experiments.

\*\* Determination of inducible resistance against  $\beta$ -lactam antibiotics using the five-disk test. (NT: not tested, +: inducible resistance)

investigator have shown that after partial gastrectomy, some patients have persistent *H. pylori* infection for five years or more<sup>7,8</sup>. Our results suggested that *H. pylori* strains (helical forms) could come into contact with other bacteria in the remnant stomach. Generally, drug resistance genes such as transposons and drug resistance plasmids confer resistance to a wide spectrum of drugs and cause serious problems in the clinical setting. It has been shown that *H. pylori* can take up extracellular DNA and undergo conjugation<sup>10</sup>. Therefore, it is important to investigate factors in the environment of the remnant stomach that may make it easier for *H. pylori* to acquire drug resistance genes. The minimum inhibitory concentrations ( $\mu$ g/ml) of antibiotics for microorganisms isolated from the gastric juice of patients with a remnant stomach are shown in Table 2. In this study, only one of eight strains produced  $\beta$ -lactamase (KU6274) and KU6271, KU6272, and KU6273 all showed high MICs for AMP and CET. We thought that this was the result of  $\beta$ -lactamase activity, so we performed the five disk tests. The disk with IPM, a  $\beta$ -lactamase inducer, was placed at the center of the agar plate, while the AMP, CET, PIPC, and CAZ disks were placed 20 mm apart from each other. KU6271 showed a zone of inhibition around the PIPC disk proximal to the IPM disk that was uniformly blunted, while

KU6272 and KU6273 showed a zone of inhibition around the CAZ disk proximal to the IPM disk that was uniformly blunted, in agreement with the inducible nature of resistance in these strains.

We examined the effects of mixed cultures of *E. coli* and *H. pylori* and could not detect any transconjugant from *E. coli* to *H. pylori*. However, we found that *H. pylori* grew on the plate containing AMP as did *E. coli*. Since *H. pylori* strain did not produce  $\beta$ -lactamase, and its MIC for AMP was less than 0.063  $\mu$ g/ml, it would appear that the  $\beta$ -lactamase produced by *E. coli* allowed *H. pylori* to survive the plate containing AMP.

At present, two antibiotics (clarithromycin and amoxicillin (AMPC) or metronidazole) and a proton pump inhibitor are used to eradicate *H. pylori*. AMPC may exhibit bactericidal activity for bacteria isolated from the remnant stomach. Accordingly, if bacteria in the remnant stomach, capable of producing constitutive or inducible  $\beta$ -lactamases, high levels of  $\beta$ -lactamase may be released in response to treatment with AMPC. Thus, *H. pylori* may be able to survive in the remnant stomach despite AMPC treatment.

In the future, it is possible that  $\beta$ -lactam antibiotics will be not effective against some *H. pylori* strains, which

acquire drug resistance *via* the transfer of drug-resistance genes in plasmids, transposons, and chromosomal elements of these organisms. It is important to monitor drug sensitivity of *H. pylori*.

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