Isolation and Characterization of Tetracycline-Resistant Clinical Isolates of *Helicobacter pylori*

DONG H. KWON,^{1*} J. J. KIM,² M. LEE,¹ Y. YAMAOKA,¹ M. KATO,³ M. S. OSATO,¹
F. A. K. EL-ZAATARI,¹ AND DAVID Y. GRAHAM¹

Department of Medicine, Baylor College of Medicine, and Veterans Affairs Medical Center, Houston, Texas 77030¹;

Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul,

Korea²; and Department of Endoscopy, Hokkaido University School of Medicine, Sapporo, Japan³

Received 14 June 2000/Returned for modification 17 July 2000/Accepted 2 August 2000

Tetracycline is an important component of combination therapies for *Helicobacter pylori* eradication. Twenty-nine tetracycline-resistant isolates requiring MICs ranging from 4 to 16 µg/ml were isolated from Korean (22 of 460) and Japanese (7 of 105) patients. Interestingly, all of the 29 tetracycline-resistant isolates exhibited cross-resistance to metronidazole, and the cross-resistance was transferred to tetracycline-sensitive *H. pylori* strains.

Helicobacter pylori infection is one of the most common infections worldwide and is etiologically associated with chronic gastritis, duodenal ulcers, gastric ulcers, gastric adenocarcinoma, and primary gastric lymphoma (3, 12, 13). Clinical experience has demonstrated that the eradication of *H. pylori* from infected patients is not easy and that the difficulty is mostly due to the lack of patient compliance with drug regimens and the development of antibiotic-resistant *H. pylori* (4). Tetracyclines are a family of broad-spectrum antibiotics that have been widely used for the treatment of bacterial infections since the 1950s. Extensive and widespread therapeutic use of tetracyclines in human and veterinary medicine and their use as growth promoters in animal feeds have resulted in tetracycline resistance in almost all bacterial genera, which has reduced the therapeutic usefulness of the tetracyclines (2, 14, 15, 16). For *H. pylori*, two cases of tetracycline-resistant isolates have been reported (6, 9), but no further information is currently available.

In order to understand the prevalence of antibiotic resistance among clinical *H. pylori* isolates, we have performed surveillance of antibiotic resistance using *H. pylori* obtained from Korean ($n = 460$) and Japanese ($n = 105$) patients since 1994. The patients from Japan had not received any previous *H. pylori* therapies (i.e., pretreatment isolates), but the patients from Korea received dual or triple anti-*H. pylori* therapies (i.e., posttreatment isolates). *H. pylori* strains were routinely culture on brain heart infusion (BHI) (Difco, Detroit, Mich.) agar plates and maintained as described previously (7). MIC measurements for metronidazole, clarithromycin, tetracycline, and amoxicillin were performed as described earlier (7). The resistance breakpoints used for metronidazole and clarithromycin were MICs of >8 (11) and >1 µg/ml (8), respectively. Since the resistance breakpoints for tetracycline and amoxicillin are not established for *H. pylori*, we defined resistance as MICs of >2 µg/ml for tetracycline and >8 µg/ml for amoxicillin (9).

All the tetracycline-resistant isolates (7 of 105; 6.7%) from the Japanese patients and 20 of the tetracycline-resistant isolates (22 of 460; 4.9%) from the Korean patients remained stable during stability tests (Table 1). However, two Korean resistant strains reverted to sensitive, as was previously shown

for other antibiotic resistances in *H. pylori* (6). The proportion of metronidazole-resistant *H. pylori* isolates was higher for Korean patients (40.4%) than for Japanese patients (23.8%), whereas clarithromycin resistance was more common in isolates from Japanese patients (15.2%) than in isolates from Korean patients (5.3%). Amoxicillin resistance was not seen in isolates from patients from either country. The higher frequency of metronidazole resistance and lower frequency of clarithromycin resistance in the isolates from Korean patients than in the isolates from Japanese patients probably reflect the relative use of those agents in the two countries. Of interest, all of the 29 tetracycline-resistant strains were also resistant to metronidazole (MIC ≥ 8 µg/ml) (Table 1). The cross-resistance of tetracycline and metronidazole was confirmed by culturing the cells on 5% horse blood BHI agar plates containing 2 to 8 µg of tetracycline per ml and 4 to 16 µg of metronidazole per ml for 3 days.

To examine whether the tetracycline resistance determinant(s) could be transferred among clinical *H. pylori* isolates, total genomic DNA purified from two tetracycline-resistant *H. pylori* strains (KH84B and KH179A) (MICs = 16 µg/ml) was introduced into two tetracycline-sensitive *H. pylori* type strains, ATCC 43629 and ATCC 700392 (MIC = 0.5 µg/ml), by natural transformation as described by Haas et al. (5). To avoid the selection of spontaneous tetracycline-resistant colonies, recipient cells for natural transformation were adjusted to approximately 10^7 cells/ml with 1 to 3 µg of genomic DNA and spread on BHI agar plates containing 2 µg of tetracycline per ml. In parallel, a negative control was also included in the same experiment by using the same number of cells without adding DNA. The transformation frequencies were 1.2×10^{-5} (ATCC 700392 with genomic DNA from KH84B), 0.7×10^{-5} (ATCC 700392 with genomic DNA from KH179A), 0.2×10^{-5} (ATCC 43629 with genomic DNA from KH84B), and 0.5×10^{-5} (ATCC 43629 with genomic DNA from KH179A). However, the negative control did not show any colonies. The phenotypic nature of the transformed strains was analyzed using 12 colonies of each strain. Forty transformed colonies (10 colonies from each transformed ATCC 700392 and ATCC 43629 strain) were used to measure the MICs of tetracycline, metronidazole, amoxicillin, and clarithromycin. The tetracycline MICs for all of the transformed colonies were 16 µg/ml, which was identical to those for the parental strains. Importantly, the metronidazole resistance phenotype was also transferred to the

* Corresponding author. Mailing address: Rm. 3A-320 (111D), Veterans Affairs Medical Center, 2002 Holcombe Blvd., Houston, TX 77030. Phone: (713) 794-7276. Fax: (713) 795-4471. E-mail: dkwon@bcm.tmc.edu.

TABLE 1. Antibiotic resistance stability of tetracycline-resistant *H. pylori* strains

<i>H. pylori</i> strain	MIC ($\mu\text{g/ml}$) ^a			
	Tetracycline ^b	Metronidazole	Amoxicillin	Clarithromycin
Type strains				
ATCC 700392	0.5 (0.25)	4 (4)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
ATCC 43629	0.5 (0.25)	1 (1)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
Strains isolated from Korean patients ^c				
KH55A	4 (4)	8 (16)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH69A	4 (4)	32 (16)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH84B	16 (16)	32 (32)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH87B	8 (8)	>32 (>32)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH95B	4 (4)	>32 (>32)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH100A	4 (8)	16 (16)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH154B	4 (0.25)	8 (16)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH161A	4 (8)	8 (16)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH164B	4 (4)	32 (32)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH179A	16 (16)	16 (16)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH185A	8 (8)	16 (32)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH222A	4 (4)	32 (32)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH250A	4 (0.25)	16 (16)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH259A	4 (4)	8 (8)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH292B	4 (8)	8 (16)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH294A	4 (8)	32 (32)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH299A	4 (4)	8 (16)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH330A	4 (8)	16 (16)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH400A	8 (16)	8 (16)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH422B	4 (4)	8 (16)	0.5 (0.5)	2 (2)
KH430B	4 (4)	32 (16)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH439A	4 (4)	32 (32)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH453A	4 (4)	32 (32)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
KH461A	4 (4)	8 (8)	2 (2)	≤ 0.25 (≤ 0.25)
Strains isolated from Japanese patients				
JH34A	4 (4)	8 (8)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
JH86A	4 (4)	16 (16)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
JH86B	4 (4)	16 (16)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
JH98A	4 (4)	8 (8)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
JH223A	4 (4)	8 (8)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
JH244B	4 (4)	32 (32)	0.5 (0.5)	≤ 0.25 (≤ 0.25)
JH269B	4 (4)	16 (16)	0.5 (0.5)	≤ 0.25 (≤ 0.25)

^a Values in parentheses are MICs for the same *H. pylori* strains after storage at -80°C for 1 month.

^b The distribution of tetracycline MICs for the Korean isolates ($n = 460$) was as follows: ≤ 0.5 , $n = 344$; 1.0, $n = 74$; 2.0, $n = 20$; 4.0, $n = 12$; 8.0, $n = 7$; and 16.0, $n = 3$. The distribution for the Japanese isolates ($n = 105$) was as follows: ≤ 0.5 , $n = 73$; 1.0, $n = 20$; 2.0, $n = 5$; and 4.0, $n = 7$.

^c Isolates with designations ending in A were isolated from the antrum; isolates with designations ending in B were isolated from the corpus.

two type strains (also requiring MICs of 16 $\mu\text{g/ml}$). There was no change in susceptibility to amoxicillin and clarithromycin compared to that of the parental strains (Table 2).

Patients from both countries had relatively high frequencies of tetracycline-resistant *H. pylori* (4.9 and 6.7% for Korean and Japanese patients, respectively). The MICs of tetracycline for the 29 strains with stable tetracycline resistance were 4 to 16 $\mu\text{g/ml}$. However, when the strains grew on low-level tetracycline-containing agar plates before the MICs were measured, the MICs increased more than twofold, suggesting that tetracycline-resistant strains would compromise therapies containing tetracycline. All the tetracycline-resistant strains showed cross-resistance to metronidazole, requiring MICs ranging from 8 to ≥ 32 μg of metronidazole per ml, and the metronidazole resistance remained stable. In addition, the tetracycline resistance from the clinical isolates was always transferred together with metronidazole resistance to the tetracycline-sensitive type strains and remained stable, as shown for the parental tetracycline-resistant strains. These observations imply that resistance to tetracycline and metronidazole from the tetracycline-resistant *H. pylori* clinical isolates is transferable to the

TABLE 2. Antibiotic resistance patterns of transformed *H. pylori* strains

<i>H. pylori</i> strain	MIC ($\mu\text{g/ml}$)			
	Tetra-cycline	Metro-nidazole	Amoxi-cillin	Clari-thromycin
Type strains				
ATCC 700392	0.5	4	0.5	≤ 0.25
ATCC 43629	0.5	1	0.5	≤ 0.25
Clinical isolates				
KH84B	16	32	0.5	≤ 0.25
KH179A	16	16	0.5	≤ 0.25
Transformed type strains				
ATCC 700392/KH84B ^a	16	16	0.5	≤ 0.25
ATCC 700392/KH179A ^b	16	16	0.5	≤ 0.25
ATCC 43629/KH84B ^a	16	16	0.5	≤ 0.25
ATCC 43629/KH179A ^b	16	16	0.5	≤ 0.25

^a Transformed by genomic DNA purified from *H. pylori* KH84B.

^b Transformed by genomic DNA purified from *H. pylori* KH179A.

sensitive *H. pylori* strains and also suggest that tetracycline resistance may be associated with metronidazole resistance but not vice versa. Interestingly, tetracycline-resistant *H. pylori* strains isolated by other investigators also showed cross-resistance to metronidazole (1, 9). The metronidazole resistance mechanism has been reported to occur with alterations in the *rdxA*, *frxA*, and/or *fdxB* gene of *H. pylori* (7). The mechanism of tetracycline resistance has not been reported for *H. pylori*, although the tetracycline resistance mechanism has been extensively studied for *E. coli* and other bacteria (2, 14, 15, 16). It is not clear whether the cross-resistance mechanism is due to a known metronidazole resistance mechanism and an unknown tetracycline resistance mechanism or if it is a part of multidrug resistance mechanisms, as in other gram-negative bacteria (10). In any case, the emergence of a new transferable antibiotic resistance among clinical isolates represents a major threat to current *H. pylori* eradication therapies. Currently, we are attempting to elucidate the possible mechanism(s) for the dual antibiotic resistance seen in these strains.

This work was supported in part by the U.S. Department of Veterans Affairs.

REFERENCES

1. Boyanova, L., I. Stancheva, Z. Spassova, N. Katarov, I. Mitov, and R. Koumanova. 2000. Primary and combined resistance to four antimicrobial agents in *Helicobacter pylori* in Sofia, Bulgaria. *J. Med. Microbiol.* **49**:415–418.
2. Chopra, I., P. M. Hawkey, and M. Hinton. 1992. Tetracycline, molecular and clinical aspects. *J. Antimicrob. Chemother.* **29**:245–277.
3. Crump, M., M. Gospodarowicz, and F. A. Shepherd. 1999. Lymphoma of the gastrointestinal tract. *Semin. Oncol.* **26**:324–337.
4. Graham, D. Y., W. A. de Boer, and G. N. Tytgat. 1996. Choosing the best anti-*Helicobacter pylori* therapy: effect of antimicrobial resistance. *Am. J. Gastroenterol.* **91**:1072–1076.
5. Haas, R., T. F. Meyer, and J. P. M. van Putten. 1993. Aflagellated mutants of *Helicobacter pylori* generated by genetic transformation of naturally competent strains using transposon shuttle mutagenesis. *Mol. Microbiol.* **8**:753–760.
6. Han, S.-R., S. Bhakdi, M. J. Maeurer, T. Schneider, and S. Gehring. 1999. Stable and unstable amoxicillin resistance in *Helicobacter pylori*: should antibiotic resistance testing be performed prior to eradication therapy? *J. Clin. Microbiol.* **37**:2740–2741.
7. Kwon, D.-H., F. A. K. El-Zaatari, M. Kato, M. S. Osato, R. Reddy, Y. Yamaoka, and D. Y. Graham. 2000. Analysis of *rdxA* and involvement of additional genes encoding NAD(P)H flavin oxidoreductase (FrxA) and ferredoxin-like protein (FdxB) in metronidazole resistance of *Helicobacter pylori*. *Antimicrob. Agents Chemother.* **44**:2133–2142.
8. Mégraud, F., N. Lehn, T. Lind, E. Bayerdörffer, C. O'Morain, R. Spiller, P. Unge, S. V. Van Zanten, M. Wrangstadh, and C. F. Burman. 1999. Antimicrobial susceptibility testing of *Helicobacter pylori* in a large multicenter trial: the MACH 2 study. *Antimicrob. Agents Chemother.* **43**:2747–2752.
9. Midolo, P. D., M. G. Korman, J. D. Turnidge, and J. R. Lambert. 1996. *Helicobacter pylori* resistance to tetracycline. *Lancet* **347**:1194–1195.
10. Nikaido, H. 1996. Multidrug efflux pumps of gram-negative bacteria. *J. Bacteriol.* **178**:5853–5859.
11. Osato, M. S., R. Reddy, and D. Y. Graham. 1999. Metronidazole and clarithromycin resistance amongst *Helicobacter pylori* isolates from a large metropolitan hospital in the United States. *Int. J. Antimicrob. Agents* **12**:341–347.
12. Parsonnet, J. 1998. *Helicobacter pylori*. *Infect. Dis. Clin. N. Am.* **12**:185–197.
13. Parsonnet, J. 1998. *Helicobacter pylori*: the size of the problem. *Gut* **43** (Suppl. 1):S6–S9.
14. Roberts, M. C. 1996. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. *FEMS Microbiol. Rev.* **19**:1–24.
15. Schnappinger, D., and W. Hillen. 1996. Tetracyclines: antibiotic action, uptake, and resistance mechanisms. *Arch. Microbiol.* **165**:359–369.
16. Speer, B. S., N. B. Shoemaker, and A. A. Salyers. 1992. Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. *Clin. Microbiol. Rev.* **5**:387–399.