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Clinical Application of the DiversiLab Microbial Typing System Using Repetitive Sequence-Based PCR for Characterization of *Helicobacter pylori* in Japan

Norihito Morimoto,[1] Hiroaki Takeuchi,[2]* Yoshie Nishida,[1,2] Mie Morisawa,[1] Tomoe Yoshikawa,[1] Tamae Morita,[1] Miyuki Morimoto,[1] Chizuko Sugimoto,[2] Yoshihisa Matsumura,[2] and Tetsuro Sugiura[1,2]

[1]*Department of Clinical Laboratory, Kochi Medical School Hospital, Kochi University, Kohasu, Oko-cho, Nankoku, Kochi, Japan*

[2]*Clinical Laboratory Medicine, Kochi University School of Medicine, Kochi, Japan*

We evaluated the DiversiLab (DL) system with universal primers, a semiautomated repetitive extragenic palindromic sequence-based polymerase chain reaction (PCR) (rep-PCR) system, for the characterization of *Helicobacter pylori* in Japan. All 135 isolates from Japanese patients with gastric cancer (GC, $n = 55$) or non-GC ($n = 80$) were used and subjected to the drug susceptibility examinations (amoxicillin, AMPC; metronidazole, MNZ; and clarithromycin, CAM) by E-test. There were 28 MNZ-resistant (20.7%), 35 CAM-resistant (25.9%), and 16 MNZ/CAM-resistant (11.9%) isolates. DL rep-PCR fingerprinting analysis at the level of 95% similarity revealed five major groups (A–E) and the other including 45 isolates. The occupation rates of GC-derived isolates in groups B (54.2%) and E (58.8%) were higher than in the other groups: A (26.7%), C (28.6%), D (30.0%), and the other (40.0%). Relative higher occupation rates of drug resistants, such as MNZ-, CAM- and double MNZ/CAM-resistant isolates, were observed in groups B (45.8%), C (42.6%), and D (40%). Five of eight GC-derived isolates with MNZ/CAM resistance were significantly assigned to group B ($P = 0.0312$, χ^2-test). These results suggest that the isolates classified in group B have a potential to contribute to the development of severe gastric disorders. The DL system, rapid and high sensitive technology, would be widely available in clinical laboratory for pathological and epidemiological analyses even in *H. pylori*. J. Clin. Lab. Anal. 29:250–253, 2015. © 2014 Wiley Periodicals, Inc.

Key words: *Helicobacter pylori*; genotyping; rep-PCR; DiversiLab Microbial Typing System; gastric cancer; antibiotic resistant

Helicobacter pylori colonizes the human stomach causing a variety of diseases. The isolates obtained from *H. pylori* associated diseases are used to establish bacterial pathogenity and/or the risk factors as bacterium–host interaction. However, these findings are insufficient to explain the development of all *H. pylori* associated diseases. Thus, developing a tool for interpretation of the risk factors and clinical pathology of *H. pylori* associated diseases is important. Repetitive sequence-based PCR (rep-PCR) assay determines the similarity among individual bacteria or strains at the level of genomic variation via analysis of the repetitive sequences throughout the genomes. Rep-PCR greatly enhances a reproducibility and strain discrimination compared with other PCR-based platforms (1,2). Thus, rep-PCR is recognized as an effective method (3) for epidemiological analysis and investigation of the relationship between microorganisms and their related disorders. Rep-PCR with specific primers has grouped *H. pylori* isolates from duodenal ulcer or simple gastritis

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*Correspondence to: Hiroaki Takeuchi, Department of Clinical Laboratory Medicine, Kochi Medical School, Kochi University, Kohasu, Oko-cho, Nankoku, Kochi 783-8505, Japan. E-mail: htake@kochi-u.ac.jp

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patients into distinct clusters (4, 5), suggesting that this method is useful to provide a strain characteristics of risk potential and/or pathogenesis of diseases. The DiversiLab (DL) system (bioMerieux, Marcy l'Etoile, France) is a rep-PCR technique that offers semiautomated, easy-to-use, high-throughput, and rapid bacterial strain typing (6,7). However, there is no *H. pylori* genotyping data available using the DL system. In this study, we examined the discrimination of *H. pylori* isolates from gastric cancer (GC) and non-cancer patients and evaluated whether DL system can be used to determine the potential risk factor in the clinical laboratory.

This study was retrospectively analyzed with 135 *H. pylori* isolates collected in different geographical locations, including 55 and 80 isolates from GC (46–86 years, average 67.7 years) and non-GC (12–84 years, average 56.9 years), respectively. All isolates were confirmed as *H. pylori* by routine microbiological and genetic examinations. *Helicobacter pylori* was cultured on Brucella broth agar plates containing 10% horse serum and 10 μg/ml of vancomycin at 37°C under microaerobic conditions (10% CO_2; (8)). Genomic DNA was extracted using the UltraClean microbial DNA isolation kit (MO BIO Laboratories, Carlsbad, CA), and the regions of interest were amplified using the DL Bacterial kit with universal primers according to the manufacturer's instructions. The PCR amplicons were applied to the DL system that generated a genetic pattern using a DL Lab-chip kit and Agilent B2100 bioanalyzer (Agilent Technologies, Germany). The genetic patterns were compared and dendrograms based on the pattern similarity were generated by the DL software (version 3.3). An interactive report consisting of the dendrogram, electropherogram, virtual gel images, scatter plots, and selectable demographic fields was automatically generated. Antibiotic susceptibility was tested by E-test® (bioMérieux, France) for amoxicillin (AMPC), metronidazole (MNZ), and clarithromycin (CAM) via a determination of minimum inhibitory concentration (MIC). The bacterial suspension adjusted to a turbidity of 3–4 McFarland was spread on a Brucella broth agar plate containing 10% horse serum and each antibiotic stick was placed on the plates, followed by culture for 72 hr. The breakpoints used to define the resistance were as follows: AMPC > 0.5 μg/ml, MNZ > 8 μg/ml, and CAM > 1 μg/ml (9).

All 135 *H. pylori* isolates including 55 GC-derived and 80 non-GC-derived isolates were clustered into five major groups (A–E) and the other including 45 individual strains by pattern typing by DL at the level of 95% similarity (Fig. 1). A total of 90 isolates belonged to the major groups consisting of 14–24 isolates in each group. We repeated rep-PCR typing using DNAs of *H. pylori* ATCC 26695 and two clinical strains at least three times, and demonstrated that each similarity was ≥98.5% (data not shown). Thus, the isolates representing ≥98.5% pattern similarity could be considered as identical level in this rep-PCR typing. We used an ATCC 26695 strain in every experiments as an internal control to monitor the reliability of the assay. The occupation rates of GC-derived isolates in group B (54.2% [13/24]) and group E (58.8% [10/17]) were higher than those of A (26.7% [4/15]), C (28.6% [4/14]), D (30.0% [6/20]), and the other (40.0% [18/45]). The antibiotic susceptibility of all 135 isolates demonstrated 20.7% (28/135), 25.9% (35/135), and 11.9% (16/135) in MNZ, CAM, and double MNZ/CAM resistance, respectively. No AMPC-resistant isolate was found. There was no significant relationship between drug susceptibility and rep-PCR typing. Interestingly, as for the 16 MNZ/CAM resistance, it showed the tendency that such double drug-resistant strains were intensively classified to groups B or D (Fig. 1). Furthermore, eight GC-derived isolates with MNZ/CAM resistance were found, of which five isolates were assigned to group B with significance ($P = 0.0312$, χ^2-test).

Generally, genotyping in epidemiology and clinical pathology improves our understanding of genetic variation/evolution, leading to appropriate treatment of nosocomial infection and infectious diseases. The DL system developed, a rep-PCR tool, is widely applied and good performance of DL system was reported for subtyping of several pathogens (10,11). Rep-PCR with primers specific to certain genes of *H. pylori* documented that there was no significant difference of genotyping of isolates from between patients with GC or duodenal ulcer (12). In contrast, the genotyping in *H. pylori* isolates by pulsed-field gel electrophoresis and arbitrary primer PCR reflected the characteristics of antibiotic susceptibility (13). To the best of our knowledge, there is no assessment regard to bacterial discrimination and genotyping of *H. pylori* using the DL system.

In this study, all 135 *H. pylori* isolates were subjected to a universal kit of the DL system composed of specific fingerprint kits for several bacteria except of *H. pylori*. There is no universal tethering for identification of *H. pylori* using the universal kit of the DL system. Thus, we investigated the reliability and reproducibility of this assay using DNAs of *H. pylori* ATCC 26695 and two clinical isolates, which showed ≥98.5% similarity as an identical level. Rep-PCR at the level of 95% similarity revealed that all isolates were divided into five major (A–E) and the other groups. The other group composed of 45 isolates included eight isolates with <80% similarity. On the other hand, high-similar strains (≥98.5% similarity) were found in the other as well as five major groups, leading to several minor groups. These minor groups were shown in Figure 1. These high-similar isolates were from patients with individual geography and clinical records, such as diagnosis, treatment, and hospital. Thus, it suggested that these isolates were not identical, but showed

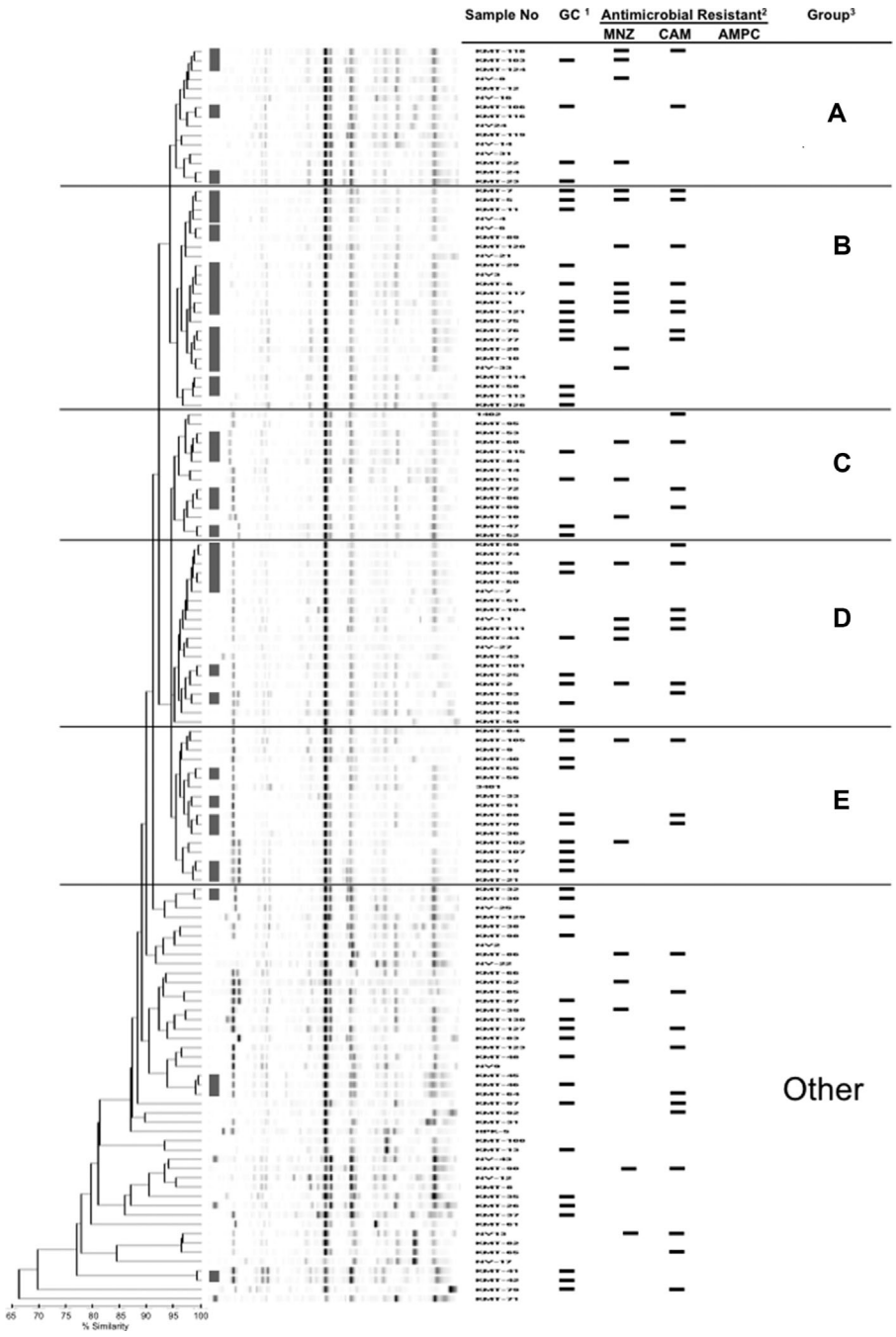

Fig. 1. Dendrogram based on rep-PCR pattern typing with 95% similarity level for 135 *Helicobacter pylori* isolates. [1]Gastric cancer (GC)-derived *Helicobacter pylori* isolates were shown by bar. [2]Drug susceptibility examination. The isolates resist to antibiotics were shown by bar. MNZ, metronidazole; CAM, clarithromycin; AMPC, amoxicillin. [3]Five major groups (A–E) and the other were provided by DL analysis. ▓: The minor groups consisted of high-similar isolates (≥ 98.5% similarity) was depicted.

extremely high similarity in this assay. We need more investigations to clear these issues (identical or not) as well as the marginal capacity of the utility.

Go et al. reported that different pattern was obtained from duodenal ulcer isolates and gastritis isolates by rep-PCR of *H. pylori* (4). The rep-PCR fingerprints showed clustering by geographic region, but not by disease presentation (12). Interestingly, our results documented that the occupation rates of GC-derived isolates were higher in group B (54.2%) and group E (58.8%) than other groups. CagA and VacA, major pathogens of *H. pylori*, are well known to relate with gastric carcinoma (14). However,

both genes were conserved in all isolates by PCR (data not shown), suggesting that the peculiar situation of the rep sequences around the both genes of the isolates in two groups (B and E) may profoundly reflect the pathogenicity of CagA and VacA. In the future, we should accumulate these data and generate rep-PCR libraries to serve as reference standards for pathogenic analysis.

Drug-resistant isolates distributed over six groups, indicating that this genotyping could not reflect the antibiotic susceptibility (MNZ, CAM, and AMPC). However, 16 MNZ/CAM-resistant isolates were found and these double drug-resistant isolates seemed to be intensively divided into group B ($n = 6$) and group D ($n = 4$). Furthermore, five of eight GC-derived isolates with double drug-resistant were significantly assigned to group B ($P = 0.0312$, χ^2-test). Therefore, the isolates in group B deserved further investigation to clarify biological characteristics in term of latent pathogenesis and multidrug resistance.

Helicobacter pylori has a capacity for high genetic diversity and infection in early childhood possibly leads to persistent colonization in the stomach with adaptation to individual hosts (15–18). Consequently, the *H. pylori* genome has been rearranged for persistent infection in the individual stomachs. Li et al. reported that the differences among the *H. pylori* strains exist in single gene allelic variants as well as in the conserved noncoding regions, such as rep sequences, throughout the entire bacterial genome (12). Thus, DL system may be available to detect such genetic alterations of *H. pylori*. DL system in laboratory will enable comprehensive genetic analysis of *H. pylori* and provide important information in clinical pathology.

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