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Nested-Polymerase Chain Reaction for the Detection of *Helicobacter pylori* Infection with Novel Primers Designed by Sequence Analysis of Urease a Gene in Clinically Isolated Bacterial Strains

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We have established a highly sensitive semi-nested PCR assay for the detection of *H. pylori* infection using gastric juice samples, which can be aspirated with disposable nasogastric tubes. The primers targeting *H. pylori* urease A gene were designed based on the sequence conservation analysis of sixteen *H. pylori* strains isolated from Japanese patients. The efficacy of the PCR assay, designated as the URA-PCR, was confirmed by *in vitro* and *in vivo* assessments. Its sensitivity was 97.5% in the gastric juice samples aspirated from forty patients with proven *H. pylori* infection, and was significantly higher than that obtained with previously described PCR assays.

The bacterium, *Helicobacter pylori* (1), plays an important role in the pathogenesis of gastritis, peptic ulcer (2), and gastric cancer (3). As a method of detecting *H. pylori* infection, the polymerase chain reaction (PCR) on gastric juice samples (4–7) has an advantage over conventional methods which require gastric biopsy specimens, such as bacterial culture and the rapid urease test, because the former can assess the infection over the entire gastric mucosa. However, previous reports using PCR on gastric juice samples (5–7), including our own (7), have failed to demonstrate its superiority to conventional methods.

We have designed novel primer sets for semi-nested PCR amplification of *H. pylori* urease gene to be used with gastric juice samples, based on the sequence analysis of clinically isolated *H. pylori* strains. In addition, we adopted the antigen-capture technique to prevent possible inhibition on PCR amplification by certain substances in the gastric juice. As a result, we have established a highly sensitive semi-nested PCR assay for the detection of *H. pylori* infection.

MATERIALS AND METHODS

Cultured H. pylori strains. Forty *H. pylori* strains had been isolated from Japanese patients and cultured at the Bacteriology Laboratory, SRL. The bacterial DNA was extracted from the culture media using SepaGene (Sanko Junyaku, Japan). In sixteen of them, the DNA sequence of urease A gene was analyzed with the dye deoxy terminator cycle sequence method using 373A DNA Sequencer (Applied Biosystems).

Gastric juice samples. Gastric juice samples were aspirated with disposable nasogastric tubes from forty Japanese patients with *H. pylori* infection proven beforehand by the bacterial culture of gastric biopsy specimens (Male/Female 25/15, age 50 ± 12 year, range 23 to 71). Their endoscopic diagnoses were: gastric and/or duodenal ulcer in 25, chronic gastritis in 13, and normal mucosa in 2. To avoid possible interference from substances in the gastric juice, *H. pylori* was first captured on rabbit anti-*H. pylori* antibody (DAKO A/S, Denmark) immobilized on polystyrene beads. After elution from the beads, DNA was extracted using the SepaGene.

PCR primer design. Several PCR primer candidates were selected based on the computer-assisted analysis of the standard urease A gene DNA sequence (GenBank accession M60398) (8). After considering the sequence conservation among the sixteen clinically-isolated strains, we chose two sense primers, A-2F2 and A-2F3, and an anti-sense primer, A-2R, as the

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optimal ones (Table 1). A novel semi-nested PCR assay, designated as URA-PCR, was thus designed with A-2F2/A-2R as the first and A-2F3/A-2R as the internal PCR primers. Its efficacy was compared with that of other PCR assays (URB-, HPU-, and HP-PCR). The semi-nested URB-PCR was designed by us targeting the urease B gene (8). The nested HPU-PCR was previously described by Clayton (9) targeting urease A gene, and the semi-nested HP-PCR, by Ho (10) targeting 16S ribosomal RNA gene. The primers were synthesized with 392 DNA Synthesizer (Applied Biosystems).

PCR amplification. PCR was performed with the automatic thermal cycler (Perkin-Elmer Cetus). A PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min: 30 cycles for the first and 20 cycles for the second PCR amplification. The PCR products were analyzed on 3%-agarose gel electrophoresis using ethidium bromide staining for visualization.

Specificity. Specificity of the novel PCR primers designed by us was evaluated with the DNA samples extracted from thirty-six bacterial species other than *H. pylori,* including two *Helicobacter* species, *H. fennelliae* and *H. cinaedi.*

Sensitivity. This was assessed by using the extracted DNA samples from the forty cultured *H. pylori* strains and the gastric juice samples obtained from the forty patients with *H. pylori* infection. The detection thresholds of PCR assays were determined by using sequentially diluted *H. pylori* DNA samples obtained from a culture medium, whose colony forming unit (CFU) density had been determined beforehand.

RESULTS

The sequence analysis of the urease A gene in the sixteen clinically isolated *H. pylori* strains revealed that some positions had frequent nucleotide displacement while others were well conserved (Table 1).{tab1}{tab1a}{tab1b} The standard nucleotides were displaced in all clinically isolated strains at 10 positions, and in more than half of the strains at 7 positions.

The primers used for the URA-PCR, A-2F2, A-2F3, and A-2R, were designed by targeting well conserved regions, and no strains had nucleotide variation in the sequences corresponding to A-2F3 or A-2R. Only two strains had one-nucleotide displacement in the sequence corresponding to A-2F2 (Table 1). The URA-PCR amplified the DNA of these two strains nonetheless efficiently.

A first primer and an internal one of the HPU-PCR (HPU1 and NHP2, respectively) covered regions with frequent nucleotide displacement in the clinically isolated strains (n.t. 2964 and 3228). This may have contributed to the low sensitivity of the HPU-PCR compared to the URA-PCR (see below).

The two PCR assays with novel primers designed by us (URA- and URB-PCR) did not amplify the DNA samples of thirty-six bacteria species other than *H. pylori,* but amplified the samples of all forty clinically isolated *H. pylori* strains, confirming their specificity and sensitivity, respectively. The detection threshold of the URA- and URB-PCR assays as determined with the sequentially diluted *H. pylori* DNA samples was substantially smaller than that of the other two PCR assays described previously (HPU-PCR and HP-PCR, Table 2).{tab2}

The efficacy of the URA-PCR was also demonstrated by assays on the gastric juice samples obtained from patients with *H. pylori* infection (Table 2 and Fig 1). The URA-PCR was the most sensitive among the three PCR assays tested, being positive in 39 of 40 samples (sensitivity 97.5%). The first-step amplification of the URA-PCR was positive only in 28 samples: the second-step one was essential in the detection of *H. pylori* in the remaining 11 samples. The only sample that was negative by the URA-PCR was also negative by the URB- or HPU-PCR. There were two samples that were negative by the URB-PCR and positive by the HPU-PCR; six samples negative by the latter and positive by the former. All of these eight samples were positive by the URA-PCR.

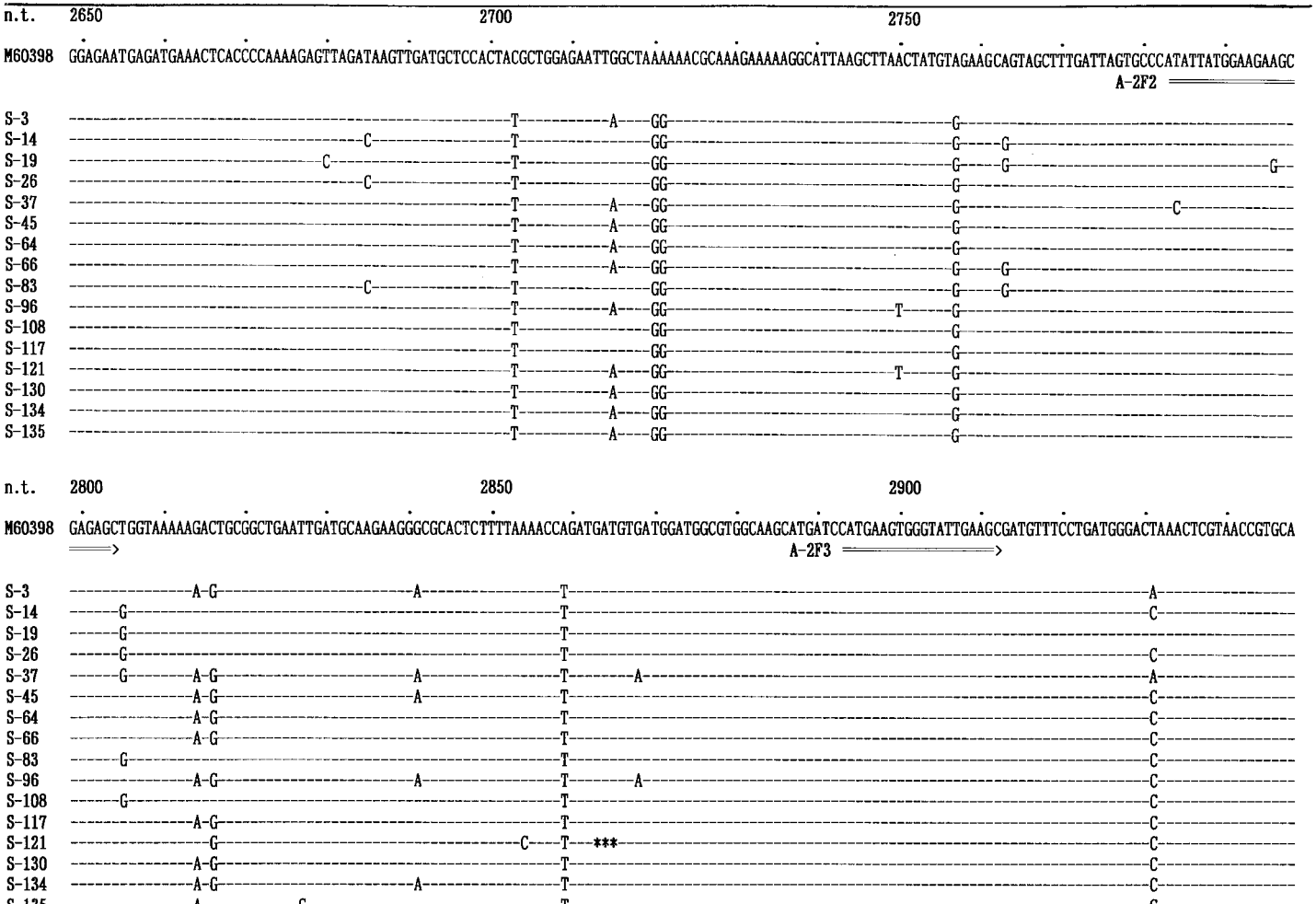
DISCUSSION

Since PCR amplifies the DNA sequences specific to targeted pathogen, it generally has excellent sensitivity and specificity provided that the primers are properly designed and contamination is carefully controlled. In this study, we have demonstrated that the URA-PCR has both excellent sensitivity and specificity for the amplification of *H. pylori* DNA. In addition, we used disposable nasogastric tubes for gastric juice aspiration to avoid contamination.

The high sensitivity of the URA-PCR was at least partly due to the good conservation of the sequences targeted by its primers (Table 1). The semi-nesting of PCR amplification was also

TABLE 1

Urease A sequence on 16 *H. pylori* Strains



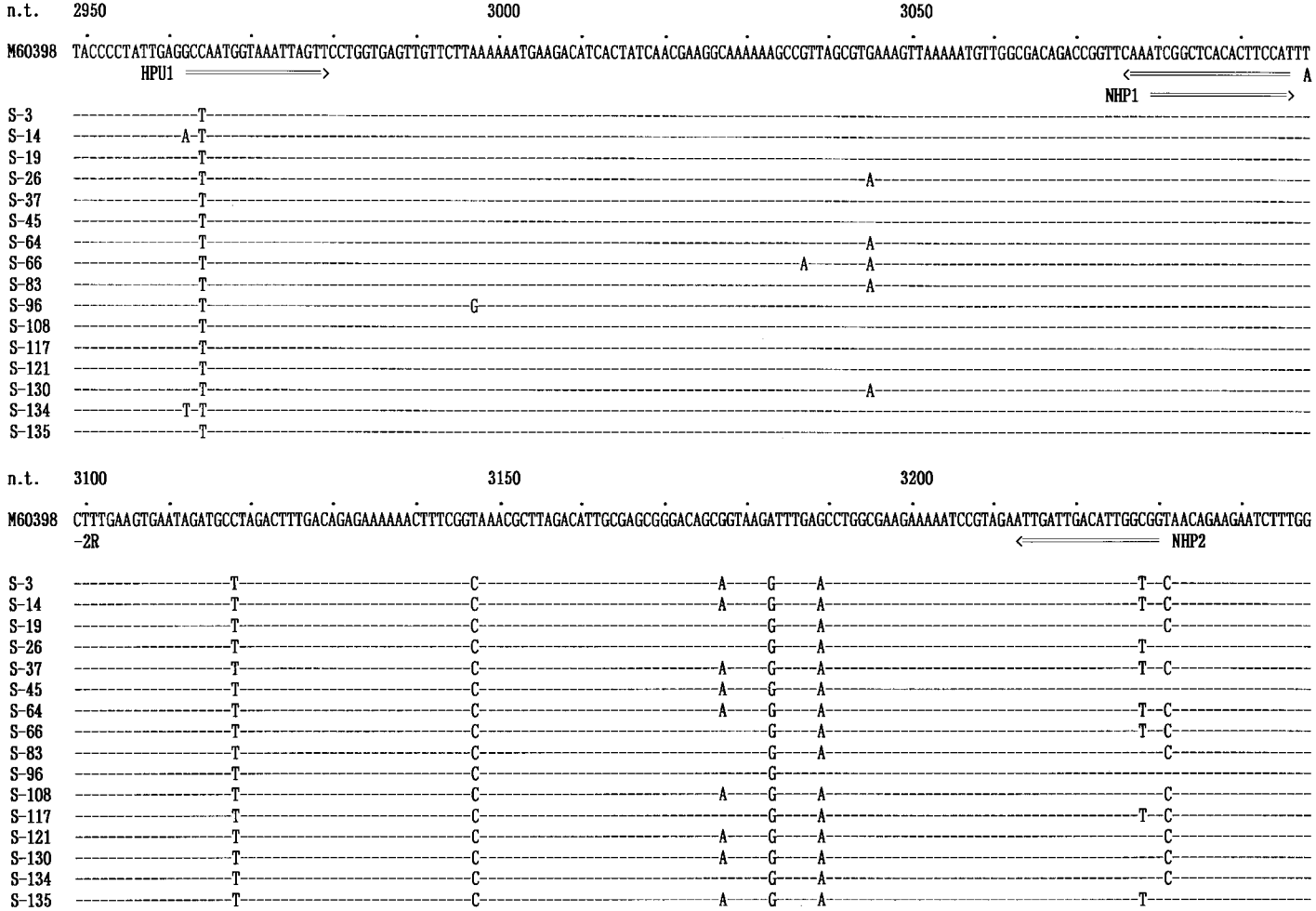
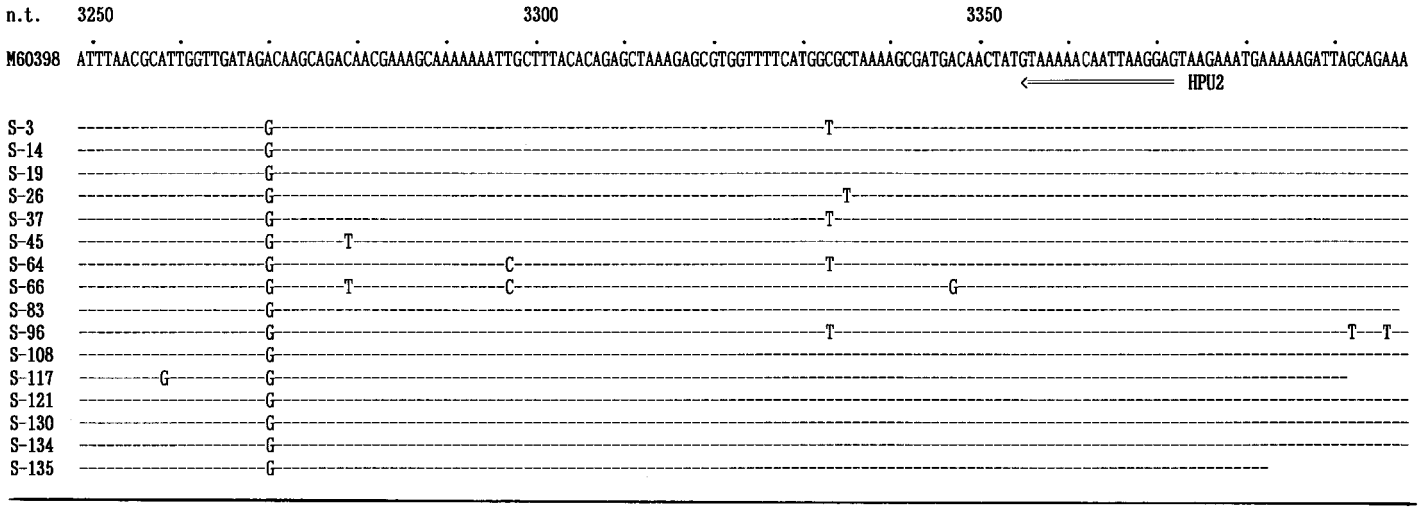

n.t. 2950 3000 3050
M60398 TACCCCTATTGAGGCCAATGGTAAATTAGTTCCTGGTGAGTTGTTCTTAAAAAATGAAGACATCACTATCAACGAAGGCAAAAAAGCCGTTAGCGTGAAAGTTAAAAATGTTGGCGACAGACCGGTTCAAATCGGCTCACACTTCCATTT
HPU1
A
NHP1
S-3 T
S-14 A T
S-19 T
S-26 T A
S-37 T
S-45 T
S-64 T A
S-66 T A A
S-83 T A
S-96 T G
S-108 T
S-117 T
S-121 T
S-130 T A
S-134 T T
S-135 T
n.t. 3100 3150 3200
M60398 CTTTGAAGTGAATAGATGCCTAGACTTTGACAGAGAAAAAACTTTCGGTAAACGCTTAGACATTGCGAGCGGGACAGCGGTAAGATTTGAGCCTGGCGAAGAAAAATCCGTAGAATTGATTGACATTGGCGGTAACAGAAGAATCTTTGG
-2R
NHP2
S-3 T C A G A T C
S-14 T C A G A T C
S-19 T C G A C
S-26 T C G A T
S-37 T C A G A T C
S-45 T C A G A
S-64 T C A G A T C
S-66 T C G A T C
S-83 T C G A C
S-96 T C G
S-108 T C A G A C
S-117 T C G A T C
S-121 T C A G A C
S-130 T C A G A C
S-134 T C G A C
S-135 T C A G A T

TABLE 1—*Continued*



Nucleotides different from those in the standard sequence (GenBank M60398) were Shown. * deletion. URA-PCR: 1st A-2F2/A-2R, 2nd A-2F3/A-2R. HPU-PCR: 1st HPU1/HPU2, 2nd NHP1/NHP2.

TABLE 2
Efficacy of PCR Assays

Sample primer set	Culture media (threshold; CFU)	Gastric juice (No. of positives in 40 samples)
URA	1–3	39(97.5%)*
URB	1–3	35(87.5%)*
HPU	10–30	31(77.5%)*
HP	10–30	Not done

*Detection rates were significantly different among the assays ($P < 0.05$ by χ^2 test).

essential for its high sensitivity. In contrast, two of the primers of HPU-PCR, HPU1 and NHP2, targeted regions where nucleotide variation was frequent (Table 1). Although the variation was found on 5′ side and thus would not much affect PCR amplification compared to variation on 3′ side, the difference of conservation may still have contributed to the difference of sensitivity between the two PCR assays.

We believe that PCR assays on gastric juice samples with properly designed primers would be much preferable for the detection of *H. pylori* infection to methods using biopsy specimens in that the former can detect the infection over the entire gastric mucosa. Furthermore, gastric juice samples can be aspirated with nasogastric tubes, which is less invasive than gastroduodenal endoscopy, not prone to contamination, and does not require special apparatus and setting.

In our previous study using gastric juice samples (7), however, H. pylori DNA was detected by a PCR assay similar to the HPU-PCR in 68 of 92 patients (74%) whose biopsy specimens were positive by the same PCR: the sensitivity of the PCR on gastric juice samples was comparable to that of conventional biopsy specimen-based tests, culture and the rapid urease test (71%). Clayton and colleagues reported that a PCR assay on gastric biopsy specimens similar to the HPU-PCR had a higher detection rate of *H. pylori* infection than conventional tests: the PCR assay detected H. pylori DNA in 15 of 23 gastric biopsy specimens (65%), while culture and microscopy revealed the

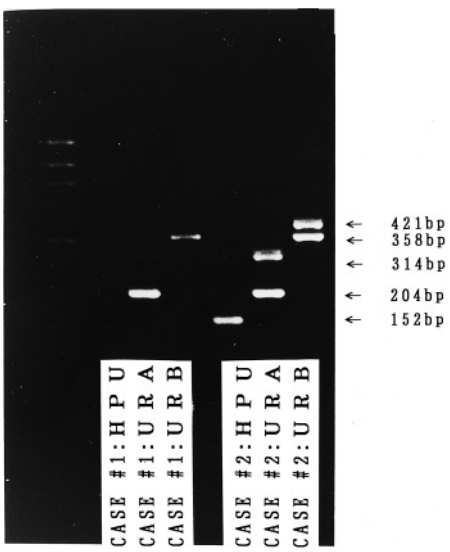


FIG. 1. Two gastric juice samples (#1 and #2) were assayed with the HPU-, URA-, and URB-PCR. In case #1, only the second PCR products of the URA-PCR (204 bp) and URB-PCR (358 bp) were positive. In case #2, the first-step PCR products of the URA-PCR (314 bp) and URB-PCR (421 bp) were also visualized, together with the second-step PCR product of the HPU-PCR (152 bp).

infection in only seven of them (30%) (8). These results indicated that the HPU-PCR assay cannot surpass biopsy specimen-based conventional tests if the former is used on gastric juice samples.

However, the data in this text have demonstrated that our novel URA-PCR is more efficient than previous PCR assays, including the HPU-PCR, and, therefore, the URA-PCR assay is possibly more efficient than the conventional methods even if using gastric juice samples. In fact, we have examined ten cases during the same period as this study whose biopsy specimens were negative for *H. pylori* by culture or the rapid urease test but gastric juice samples were positive by the URA-PCR assay. We are now undertaking a study that includes those patients to evaluate the clinical significance of this discrepancy.

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