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Multiplex PCR Assay for Rapid Detection and Genotyping of *Helicobacter pylori* Directly from Biopsy Specimens

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We developed and evaluated a simple, novel multiplex PCR assay for rapid detection of *Helicobacter pylori* infection and for the determination of *vacA* and *cagA* genotypes directly from gastric biopsy specimens. This assay did not require culturing of strains or extraction of DNA from biopsy samples. This multiplex PCR assay would be of particularly great value for laboratories in developing countries.

The gastric pathogen *Helicobacter pylori* causes gastritis and peptic ulcer disease and is an early risk factor for development of gastric adenocarcinoma (15). Because it is fastidious and microaerophilic, it cannot be cultured in many clinical or research laboratories in resource-poor developing countries. None of the methods to detect *H. pylori* infection, including the recently developed *H. pylori* stool antigen test (4) and PCR restriction analysis using a RNA polymerase gene (*rpoB*) (10), can give a clue to the virulence genes of *H. pylori* strains harbored by the host (6, 8, 9). Although Park et al. (13) developed a method for obtaining *H. pylori* genotype data directly from biopsy samples, it involves extraction of genomic DNA and requires several PCRs.

We report the development of a multiplex PCR assay by which *vacA* signal sequence (s1 and s2) and mid-region (m1 and m2) alleles and the presence or absence of a *cagA* gene can be detected in a single reaction, directly from gastric biopsy samples within 4 h after endoscopy, without a need for culture or genomic DNA extraction. This can save several weeks' time and expensive reagents (e.g., antibiotic, serum, or blood- and growth-supplemented media and reagents for DNA extraction) and instrumentation (e.g., double gas incubator). The other *H. pylori* virulence gene multiplex PCR reported earlier was not standardized for biopsy samples, required expensive Ready-To-Go PCR beads, and does not type *vacA* alleles (11).

A total of 79 *H. pylori* strains isolated from India (65 strains), Japan (6), Spain (5), Australia (strain SS1; mouse colonized), and the United Kingdom and United States (strains 26695 and J99; genomes fully sequenced) were used to develop the multiplex PCR assay. Fresh biopsies (total, 90) were collected randomly from patients with duodenal ulcer (41 biopsies), gastric ulcer (9), nonulcer dyspepsia (20), duodenitis (6), gastritis (4), and adenocarcinoma (7) and from three healthy volunteers for further evaluation of this method. Each person's infection status inferred from multiplex PCR was further assayed by rapid urease testing (RUT), and culture and genotype data obtained from biopsy multiplex PCR were evaluated by PCR using DNA extracted from cultured strains isolated from the respective patients, as described earlier (5). Nucleotide sequences of primers used are given in Table 1.

To find a suitable conserved part we aligned 20 full gene sequences of *cagA* of *H. pylori* deposited in the GenBank from different parts of the world and *cagA* sequences of the two reference (genome-sequenced) strains (strains 26695 and J99). Primers cag5c-F and cag3c-R were designed from conserved regions to amplify 350 bp (positions 109 to 459 of *cagA* of strain 26695) (Table 1).

For multiplex PCR from biopsies, specimens collected in microcentrifuge tubes containing 120 μl of sterile phosphate-buffered saline were vortexed vigorously for 2 min. The tubes were then boiled in a water bath for 15 min, cooled in ice, and centrifuged at 13,000 × *g* for 1 min. The supernatant was transferred to another tube, and 3 μl was added in 25-μl volumes containing 2.5 pmol of primers VAG-F and VAG-R, 25 pmol of primers VA1-F and VA1-R, 10 pmol of primers cag5c-F and cag3c-R, 0.25 mM of each deoxynucleoside triphosphate (Takara, Shuzo, Japan), 0.9 U of *Taq* DNA polymerase (Genei, Bangalore, India), and 1.5 mM of $MgCl_2$ in standard PCR buffer (Takara). Products were amplified under the following conditions: 3 min at 94°C for initial denaturation followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final round of 10 min at 72°C, in a Perkin-Elmer 9700 thermal cycler. The sensitivity assay for multiplex PCR and nucleotide sequencing was carried out as described elsewhere (12, 14).

Using this multiplex PCR, we characterized 65 previously isolated Indian strains. Of the strains, 44 carried an s1m1

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TABLE 1. Primers used for the amplification of *vacA* alleles and *cagA*

DNA region(s) amplified	Primer name	Primer sequence	Amplicon Size(s) (bp)	Reference or source
vacA s1/*vacA* s2	VAI-F VAI-R	5′-ATGGAAATACAACAAACACAC-3′ 5′-CTGCTTGAATGCGCCAAAC-3′	259/286	2
vacA m1/*vacA* m2	VAG-F VAG-R	5′-CAATCTGTCCAATCAAGCGAG-3′ 5′-GCGTCAAAATAATTCCAAGG-3′	567/642	3
cagA	cag5c-F cag3c-R	5′-GTTGATAACGCTGTCGCTTC-3′ 5′-GGGTTGTATGATATTTTCCATAA-3′	350	This study

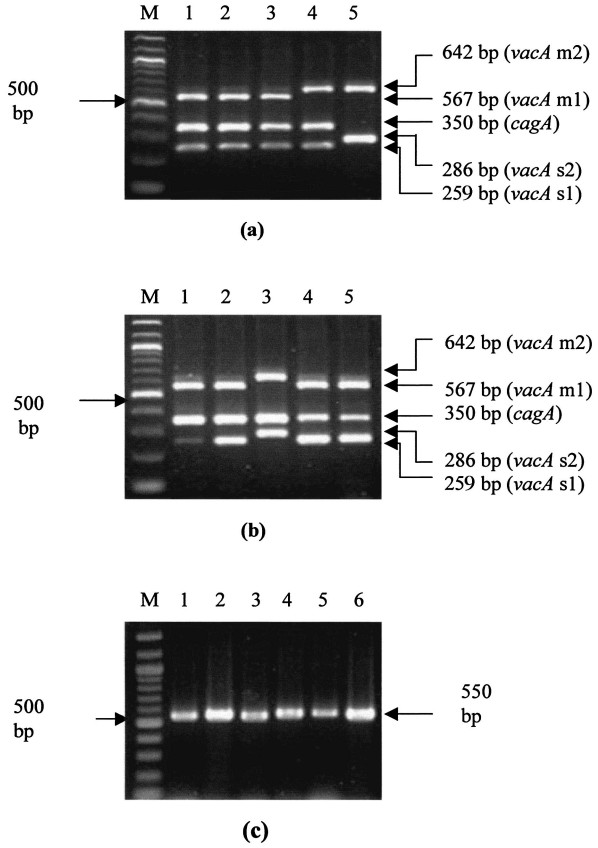

FIG. 1. Amplification of *vacA* s1, *vacA* s2, *vacA* m1, and *vacA* m2 alleles and the *cagA* gene of *H. pylori* by multiplex PCR assay. (a) Lane M, 100-bp marker (New England Biolab); lanes 1 to 5, *H. pylori* strains isolated from India. Lanes 1 to 3, s1m1 *cagA*$^+$ genotype; lane 2, s1m2 *cagA*$^+$ genotype; lane 5, genotype of s2m2 *cagA*-negative strain. (b) Lane M, 100-bp marker (New England Biolab); lanes 1 to 5, *H. pylori* strains isolated from different part of the world. Lane 1, strain HUP 77 (s1m1 *cagA*$^+$) from Spain; lane 2, strain TN2 (s1m1 *cagA*$^+$) from Japan; lane 3, strain SS1(s2m2 *cagA*$^+$) from Australia; lane 4, strain J99 (s1m1 *cagA*$^+$) from the United States; lane 5, strain 26695 (s1m1 *cagA*$^+$) from the United Kingdom. (c) Amplification of 550-bp fragment of *cag*-pathogenicity island empty site for *cagA*-negative strains discerned by multiplex PCR assay. Lanes 1 to 5, *cagA*-negative strains isolated from India; lane 6, HUP67, a *cagA*-negative strain isolated from Spain.

cagA$^+$ allelic combination, 16 carried s1m2 *cagA*$^+$, and 5 carried s2m2 and were *cagA*-negative strains (Fig. 1a). These genotypes were confirmed in each case by PCR using single primer pairs. We also accurately typed the *vacA* and *cagA* status of six strains from Japan, five strains from Spain, one mouse-colonizing strain (strain SS1) from Australia, and the two reference strains 26695 and J99 from the United Kingdom and United States, respectively, by multiplex PCR (Fig. 1b). Each of the six strains (five from India and one from Spain) that gave no *cagA* amplicon yielded the 550-bp amplicon expected of the *cag* empty site (no *cag* pathogenicity island) in PCR with primers Lunil and R5280 (12) (Fig. 1c).

Assays with the addition of boiled cultures of bacterial strains after serial dilution showed this multiplex PCR to be

TABLE 2. Sensitivity and specificity of biopsy multiplex PCR assay for the detection of *H. pylori* virulence genes in comparison with tissue rapid urease test (RUT) and culture

Test and result	No. of specimens with multiplex PCR result that was:		Total
	Positive	Negative	
RUT[a]			
Positive	41	2	43
Negative	4	43	47
Total	45	45	90
Culture[b]			
Positive	41	0	41
Negative	4	45	49
Total	45	45	90

[a] Sensitivity, 95.3%; specificity, 91.5%; positive predictive value, 91.1%.
[b] Sensitivity, 100%; specificity, 91.8%; positive predictive value, 91.1%.

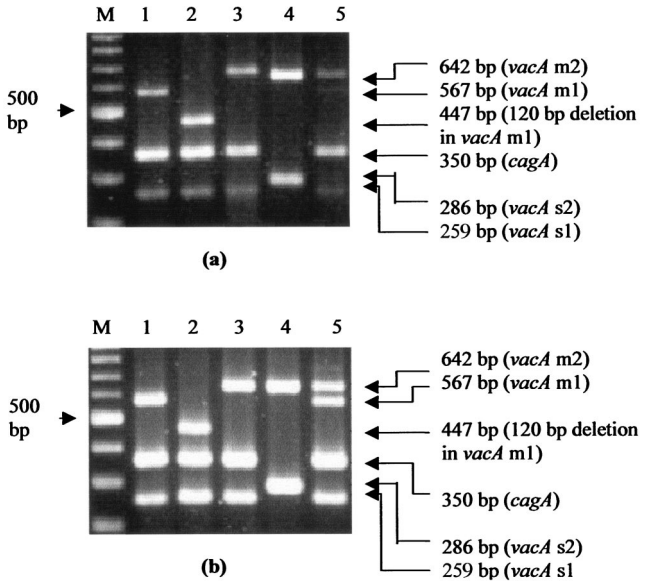

FIG. 2. Genotype of *H. pylori* obtained by multiplex PCR format. (a) Lane M, 100-bp marker (New England Biolab); lanes 1 to 5, amplification of *H. pylori* virulence genes by multiplex PCR from biopsy specimens. As shown in lane 2, biopsy 23 gave a shorter amplicon for the *vacA* middle region. (b) Lane M, 100-bp marker (New England Biolab); lanes 1 to 5, multiplex PCR results obtained from DNA extracted from strains isolated from the respective patients. As shown in lane 2, strain PCR 23 gave a shorter amplicon similar to that obtained with biopsy 23.

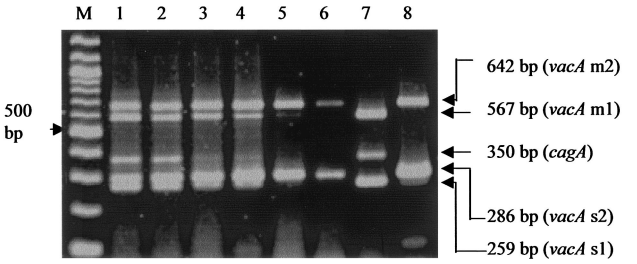

FIG. 3. Multiplex PCR assay for simulated mixed infection of strains 26695 (s1m1 *cagA*$^+$) and I-80 (s2m2 *cagA*-negative strain). Lane M, 100-bp marker (New England Biolab); lanes 1 to 6, strains 26695 and I-80 at ratios of 1:1 to 1:32; lane 7, strain 26695; lane 8, strain I-80.

highly sensitive, allowing detection of few cells per reaction. This motivated us to standardize the assay with boiled biopsy specimens.

Positive PCR was obtained with biopsies from 41 of 43 patients that had given positive results in RUT, and no PCR amplicon was obtained with biopsies from 43 of 47 patients that had given negative results in RUT (Table 2). Moreover, the method also provided preliminary *H. pylori* genotyping directly from biopsy specimens (Fig. 2). Four biopsy samples that gave positive multiplex PCR results and remained negative by culture gave positive results in RUT. Also, four RUT-negative biopsies, which gave positive results in multiplex PCR, gave positive results in culture, and the DNA extracted from cultured strains gave results identical with the genotype results obtained from their respective biopsies. Among the 41 individual patients who gave positive results for both biopsy multiplex and culture, 31 (75.6%) gave identical genotypes by multiplex PCR from biopsy and DNA extracted from cultured pooled bacterial strains. The other 10 (24.4%) gave different results, possibly due to multiple *H. pylori* strains infecting the hosts. Also, because *H. pylori* colonization in gastric mucosa is often patchy rather than uniform, the separate biopsies used for multiplex PCR and culture may harbor two different strains (1).

Amplicons for both alternative *vacA* s1/*vacA* s2 and *vacA* m1/*vacA* m2 alleles by biopsy multiplex PCR were seen with seven individuals. This outcome indicated colonization by multiple *H. pylori* strains. We also tested the ability of this multiplex PCR to identify infection by multiple *H. pylori* strains by simulating mixed infection of two bacterial strains with different genotypes (*vacA* s1/*vacA* s2 and *vacA* m1/*vacA* m2). Twofold serial dilutions were made for either strain 26695 (s1m1 *cagA*$^+$) or strain I-80 (s2m2 *cagA*-negative strain) while keeping the count of the other strain constant. Multiplex PCR performed with the serially diluted culture mixtures detected mixed genotypes, with I-80/26695 ratios ranging from 8:1 to 1:16 (Fig. 3). The ability to detect colonization by multiple *H. pylori* strains even when the two strains differ in relative abundance levels could be of great value. For many patients, suggestions of maintenance of equilibrium between *cagA*$^+$ strains and *cagA*-negative strains (7) were confirmed; at least in the Indian context, the number of *cagA*$^+$ strains in a particular gastric niche (in many cases, even in a single biopsy specimen) far exceeds the number of *cagA*-negative strains (unpublished data).

Another advantage of this multiplex PCR is that it was able to detect a deletion in *vacA*, since an amplicon for a *vacA* middle region, which is shorter than both *vacA* m1 and *vacA* m2, was obtained by biopsy multiplex PCR from one individual (Fig. 2a). The strain isolated from the same individual also showed the same *vacA* mid-region profile when subjected to multiplex PCR (Fig. 2b). Subsequent PCR using primer pairs VAm-F3–VAm-R3 and VA4-F–VA4-R (12) proved this particular *vacA* allele to be m1 (Fig. 4a and b), though the reason that a shorter amplicon was obtained in our newly designed multiplex PCR system could not be determined. Further amplification of the *vacA* middle region of this strain by PCR using primers VAG-F and VAG-R and sequencing showed a 120-bp deletion (from the second base of the codon) in this *vacA* allele when it was aligned with an m1 allele carrying the *vacA* gene sequence of strain 26695. This deletion resulted in a frameshift, which should give rise to a premature termination in peptide synthesis (Fig. 4c).

In summary, we believe the multiplex PCR developed here could be of great value in clinical microbiology and *H. pylori* population studies, especially for rapid screening of many sam-

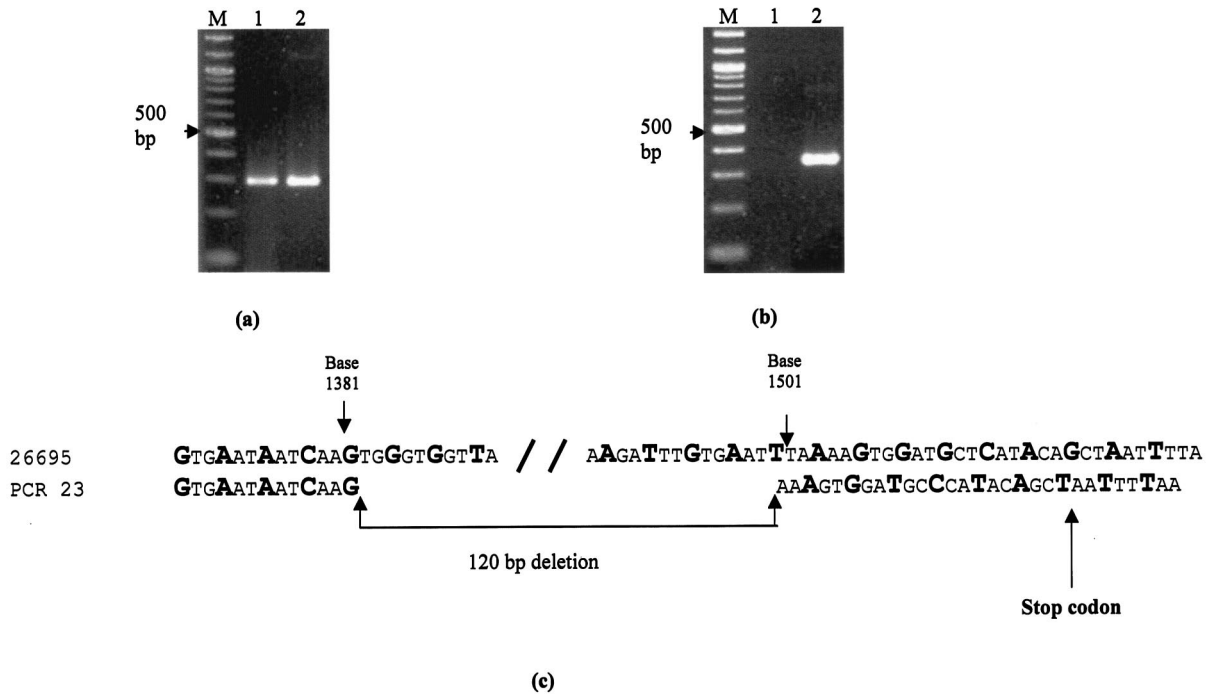

FIG. 4. Analysis of *vacA* middle region of strain PCR 23. (a) Amplification of *vacA* middle region by *vacA* m1 allele-specific primers VAm-F3–Vam-R3. M, 100-bp marker (New England Biolab); lane 1, PCR 23; lane 2, known *vacA* m1 allele containing *H. pylori* DNA. (b) Amplification of *vacA* middle region by *vacA* m2 allele-specific primers VA4-F–VA4-R. M, 100-bp marker (New England Biolab); lane 1, PCR 23; lane 2, known *vacA* m2 allele containing *H. pylori* DNA. (c) vacA sequence alignment of strain PCR 23 with strain 26695 showing that a 120-bp deletion from strain PCR 23 resulted in a frameshift after the deletion. This gives rise to a premature termination of the protein synthesis. The first bases of the codons are shown in boldface characters in a larger font.

ples to detect of *H. pylori* infection, to determine *vacA* and *cagA* status, to identify multiple-infection hosts, and to detect variant alleles.

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