MOLECULAR IDENTIFICATION AND EPIDEMIOLOGY

Identification of *Helicobacter* in gastric biopsies by PCR based on 16S rDNA sequences: a matter of little significance for the prediction of *H. pylori*-associated gastritis?

ANNIKA TIVELJUNG, K. BORCH*, J. JONASSON, S. MÅRDH†, F. PETERSSON‡
and H.-J. MONSTEIN

Divisions of Clinical Microbiology, *Surgery and †Cell Biology, Faculty of Health Sciences, University of Linköping and ‡Pathology Research Department, Ryhov Hospital, Jönköping, Sweden

The aim of the present study was to correlate molecular evidence of the presence of *Helicobacter pylori* in gastric biopsy samples, based on analysis of 16S rDNA, vacuolating toxin (*vacA*), urease A (*ureA*) and *cagA* genes, with the clinical, histological and serological findings in patients with *H. pylori*-associated gastritis. Fresh biopsy samples were collected from the gastric antrum and corpus of 22 asymptomatic volunteers with or without *H. pylori*-associated gastritis. Total DNA was extracted from the biopsy material and subjected to 16S rDNA PCR amplification, Southern blotting and 16S rDNA sequence analysis of the PCR products. The *vacA*, *ureA* and *cagA* genes were characterised by PCR amplification and Southern blot analysis. Based on partial 16S rDNA sequence analysis, DNA belonging to the genus *Helicobacter* was detected in gastric biopsy samples from 20 of 22 subjects, including seven of nine histologically and serologically normal controls. Six of 20 partial 16S rDNA sequences revealed variations within variable regions V3 and V4 that deviated from those of the *H. pylori* type strain ATCC 4350^T and, therefore, possibly represented other species of *Helicobacter*. *VacA* genes identical with those of the type strain were found predominantly in the subjects with *H. pylori* gastritis, and all the patients except one were found to be *cagA*-positive. There was no evidence of false positive PCR reactions. In conclusion, the PCR-based molecular typing methods used here were apparently too sensitive when applied to the detection of *H. pylori* in human gastric tissues. The lack of quantitative analysis makes them inappropriate as clinical tools for the diagnosis of *H. pylori*-associated gastritis, despite the fact that they provide a qualitative and sensitive tool for the detection and characterisation of *H. pylori* in the gastrointestinal tract.

Introduction

Helicobacter pylori is a micro-aerophilic gram-negative bacterium recognised as a human pathogen. It has been associated with chronic gastritis, peptic ulcer, gastric cancer and gastric mucosa-associated lymphoid tissue (MALT) lymphoma of the stomach [1]. The majority of *H. pylori*-infected individuals remain asymptomatic [2]. It has been suggested that subtypes of *H. pylori* might differ in pathogenicity [3]. Cytotoxin production (VacA) and the cytotoxin-associated gene protein

(CagA) [3, 4] are putative virulence factors. Accordingly, *H. pylori* strains have been classified into subgroups on the basis of *cagA* and *vacA* gene expression patterns [3], and different *vacA* signal sequences [5]. Several studies have revealed the presence of clonal variants in families with peptic ulcer disease [6–8]. Coccoid forms of *H. pylori*, not culturable *in vitro*, limit the use of bacterial culture for the investigation of biopsy material.

16S rDNA-based PCR methods have made it possible to detect non-culturable bacteria in human biopsy specimens [9–12]. Hoshina and co-workers identified *H. pylori* in gastric biopsy samples by PCR based on conserved and *Helicobacter*-specific 16S rDNA sequences [13]. Attempts have also been made to

Received 13 Sept. 1997; revised version accepted 24 Dec. 1997.

Corresponding author: Dr H.-J. Monstein.

characterise *H. pylori* in gastric biopsy specimens by means of reverse-transcribed PCR (RT-PCR) directed towards 16S rDNA, *cagA*, *ureA* and *ureC* mRNA [14, 15]. Comparative and competitive PCR amplification analysis of gastric biopsy samples indicate that PCR amplification methods with isolated DNA are more reliable than reverse-transcribed RNA [16, 17].

Molecular typing methods have demonstrated considerable genomic diversity among *H. pylori* isolates. In a recent study, Georgopoulos and co-workers [6] used ribotyping to characterise *H. pylori* from patients with duodenal ulcer and their spouses. Randomly amplified polymorphic DNA (RAPD) analysis has also confirmed variability in the genome of *H. pylori* [18]. Kansau and co-workers [19] used another approach to compare RAPD analysis and urease C gene (*ureC*) sequence analysis and their results indicated that both methods gave satisfactory discrimination between strains. However, *ureC* DNA sequence analysis appeared to be more reliable and reproducible [19]. In a different study, DNA from cultured *H. pylori* isolates was analysed by restriction fragment length polymorphism (RFLP) of PCR-amplified products from the *ureA* and *ureB* genes, and the results confirmed that *ureA* and *ureB* PCR-RFLP provides a novel method of discriminating between *H. pylori* strains [20].

The aim of the present study was to establish molecular typing methods without bacterial culture, which will allow the direct detection, identification and characterisation of *H. pylori* and possibly also other *Helicobacter* species in human gastric tissues, by 16S rRNA, *vacA*, *ureA* and *cagA* gene PCR amplification, Southern blot analysis and partial 16S rDNA sequence analysis of PCR-amplified products. Furthermore, this study aimed to correlate molecular evidence of the presence of *H. pylori* in the biopsy material with clinical, histological and serological findings in selected patients with or without *H. pylori*-associated gastritis.

Materials and methods

Study population

The study was approved by the local ethics committee and conducted in accordance with the Helsinki Declaration, including informed written consent. The 22 individuals examined were selected, based on histological findings and rapid urease test, from a larger (n = 501) ongoing gastroscopic screening study in a randomly selected cohort of the population of Linköping, Sweden [21]. None of the subjects in the present study had sought medical advice for abdominal complaints. There were 10 men and 12 women with median age 56 years (range 44–79) and 60 years (range 43–76), respectively. Age did not differ

significantly between men and women or between individuals with or without gastritis.

Gastroscopy and biopsy

Gastroscopy was performed after fasting overnight. Separately sterilised biopsy forceps were used for each location (corpus, antrum) in the stomach. Between gastroscopies, the forceps were treated in a Branson 3200 ultraware machine (Branson Europa BV). Both forceps and gastroscopes were washed for 30 min with EDT cleaner (ethoxybutylether 8–10%, 1,2-propylene-glycol 30–50%, proteolytic enzymes 0.5–2% and Na-alkansulphonate 3%) (Olympus Optical AB) and disinfected for 5 min with EDT disinfectant (glutaraldehyde 20–22% and ethanol 7–10%) in an Olympus Optical dishwasher for endoscopes. During gastroscopy, multiple biopsy specimens were collected from the corpus and antrum (within 3 cm of the pyloric ring) of the stomach. One biopsy specimen from each location was examined by rapid urease test (CLO test, Delta West Pty), three specimens were oriented and fixed in buffered formalin 4% and five specimens were quick frozen in liquid nitrogen and stored at –80°C until analysed. A change in colour (pH) in the rapid urease test within 12 h of placing the biopsy in the medium was regarded as indicative of the presence of *H. pylori*. After routine processing of the formalin-fixed biopsy samples, 4-µm thick sections cut perpendicular to the mucosal surface were stained with haematoxylin-eosin, Alcian blue/periodic acid-Schiff and Giemsa stain. Gastritis was classified microscopically according to the Sydney system [22, 23], including semi-quantification of the number of *H. pylori* and coccoid bacteria present on the mucosal surface or in the crypts.

Histologically, there were 13 cases with chronic gastritis and nine cases with normal morphology and without *H. pylori* in the antrum or corpus. The histological findings are summarised in Table 1. Of the 13 subjects with gastritis, one had antrum predominant (type B) gastritis with atrophy, 10 had pangastritis (type AB) (three with atrophy), and two had corpus predominant (type A) gastritis (one with atrophy).

Serological analysis

H. pylori antibodies in serum were analysed by an enzyme-linked immunosorbent assay (ELISA), as described previously (upper normal limit 5.0% of positive standard) [24]. Serological findings are summarised in Table 1.

Bacterial strain

H. pylori CCUG 17874^T (ATCC 43504^T, obtained from the Culture Collection of the University of Gothenburg, Sweden) was used as a reference strain.

Table 1. Histological and serological findings in asymptomatic individuals with *H. pylori* gastritis

Test	Subject number												
	410	418	422	430	437	453	457	467	468	479	483	485	493
<i>H. pylori</i> serology*	32.8	0.0	15.1	9.4	26.4	45.7	31.5	29.8	27.0	40.9	13.9	11.1	15.7
Corpus													
CLO test	+	-	+	-	+	+	+	+	+	+	+	+	+
<i>H. pylori</i> histology†	2	1	1	0	3	1	1	3	3	2	1	2	1
Cocci	-	-	-	-	-	-	-	-	-	-	-	-	-
Inflammation†	1	1	0	2	1	1	1	1	2	2	1	2	1
Inflammatory activity†	0	0	0	2	0	0	1	1	1	2	1	1	0
Atrophy†	0	0	0	3	0	0	0	0	1	1	0	0	0
Intestinal metaplasia†	0	0	0	3	0	0	0	0	1	2	0	0	0
Antrum													
CLO test	+	-	+	-	+	+	+	+	+	-	+	+	+
<i>H. pylori</i> histology†	3	1	3	0	3	2	1	3	2	0	2	3	2
Cocci	-	-	-	+	-	-	-	-	+	-	-	-	-
Inflammation†	2	0	2	0	2	1	1	2	2	1	2	2	1
Inflammatory activity†	1	0	1	0	1	0	1	2	2	1	1	1	1
Atrophy†	0	0	1	1	0	0	0	1	1	1	0	0	0
Intestinal metaplasia†	0	0	1	1	1	0	0	1	1	1	0	0	0
Type of gastritis	AB	A	B	A	AB	AB	AB	AB	AB	AB	AB	AB	AB

A, corpus predominant gastritis; B, antrum predominant gastritis; AB, pangastritis.
**H. pylori* antibodies expressed as percentage of positive standard (upper normal limit 5.0%); median = 26.4%; median in nine controls without gastritis 1.0 (range 0.2–14.0%) (p < 0.001, Mann-Whitney U-test).
†Grading according to the Sydney system [21, 22].

Preparation of samples for PCR amplification

Bacterial genomic DNA from the reference strain *H. pylori* CCUG 17874^T was extracted as described previously [11]. Total DNA from the human gastric biopsy specimens was prepared by extraction with the Trizol reagent-chloroform system (Gibco BRL, Life Technologies). The extraction procedure followed the manufacturer's recommendations. Briefly, the biopsy specimen (c. 5 mg) was homogenised mechanically (Polytron, Kinematica) in 0.5 ml of phenol-based Trizol reagent. The homogenate was mixed with chloroform and, after centrifugation, DNA was recovered from the phenol phase by ethanol precipitation. DNA concentration and purity were measured spectrophotometrically at OD₂₆₀ and OD₂₈₀, respectively. To exclude any possible contamination from the homogeniser, a negative control containing only the Trizol reagent was run in parallel and examined by PCR and Southern blot analysis of the PCR-amplified products.

Oligonucleotides

PCR primers were obtained from Pharmacia Biotech (Pharmacia). The 16S rDNA broad-range bacterial primers pJB-1 and p13B have been described previously [11]. Two *Helicobacter*-specific primers were designed from the variable regions V4 (HPV4) and V9 (HPV9) within the 16S rRNA gene [25]. HPV9 was used as a DNA sequencing primer and HPV4 was used as a hybridisation probe (Table 2). PCR primers and DNA hybridisation probes, directed against putative *H. pylori* virulence genes, were complementary to the urease A gene (*ureA*), the vacuolating toxin gene (*vacA*) and the cytotoxin-associated (*cagA*) gene sequences, respectively (Table 2). All primer combinations were tested in PCR amplification under the conditions described below, with human genomic DNA, to rule out any non-specific amplification.

PCR conditions

Amplifications were performed in a final volume of 50 µl containing 2 µM primer, 0.2 mM deoxyribonucleotide (dGTP, dATP, dTTP and dCTP; Boehringer-Mannheim), reaction buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM MgCl₂; pH 9.0), and 0.25 U of Taq polymerase (Pharmacia). Approximately 100 ng of total DNA were used for bacterial broad-range 16S rDNA, *ureA*, *vacA* and *cagA* amplification, respectively. PCR was performed in thin-walled 0.5-ml GeneAmp reaction tubes (Perkin Elmer), in a Minicycler PT-150 (SDS Promega) as follows: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min (30 cycles) and a final extension at 72°C for 10 min. Expected fragment sizes of the PCR amplified products are c. 450 bp for 16S rDNA, c. 680 bp for *vacA*, c. 420 bp for *ureA* and c. 350 bp for *cagA*. Negative PCR controls, containing only buffer and PCR primers, were run in parallel.

Detection and analysis of amplified PCR products

Broad-range 16S rDNA amplified PCR products were analysed by agarose gel electrophoresis and Southern blot analysis. PCR-amplified products were electrophoresed on an agarose 1.5% gel, stained with ethidium bromide and visualised under UV light. Broad-range 16S rDNA PCR products were transferred to Hybond-N membranes and hybridised to a *Helicobacter*-specific HPV4 probe, which was 5' end-labelled with [γ -³²P] dATP (3000 Ci/mmol Amersham) (1 Ci = 3.7 × 10¹⁰ Bq) and a 5' end-labelling kit (Boehringer-Mannheim). Prehybridisation and hybridisation with the HPV4 probe were carried out in SEB-buffer (0.5 M Na₂PO₄, pH 7.2, 1 mM EDTA-sodium salt; SDS 7%) at 50°C overnight. Filters were washed in 1 × SSC/SDS 0.1% for 3 × 20 min (1 × SSC = 150 mM NaCl, 15 mM sodium citrate). After the post-hybridisation step, membranes were exposed to Amer-

Table 2. Oligonucleotide primers used in PCR amplification, Southern blot, and DNA sequence analysis

Primer name	Start position in <i>H. pylori</i> DNA	DNA sequence (5'–3' orientation)	Accession number Genebank/EMBL
pJB-1* [‡]	959 [§] →	ATTGCGATGCAACGCGAAGAACCTTACC	...
p13B*	1397 [§] ←	GTGTACTAGGCCCGGGAACGTATTC	...
HPV4 [‡]	1175 [§] ←	CCTCCTTACGGAGGCAGTATCCTT	...
HPV9 [‡]	1259 [§] ←	GCTTCTCTTTGTGCACCCCAT	...
ureA-1*	2818 →	GCGGCTGAATTGATGCAAGAAGG	M60398
ureA-2 [‡]	3014 →	CTATCAACGAAGGCAAAAAGCCG	M60398
ureA-3*	3230 ←	CCGCCAATGTCAATCAATTCTACGG	M60398
vacA-1*	828 →	CAATCGTGTGGGTTCTGGAGC	S72494
vacA-2 [‡]	1337 ←	CTATTGGGTTGGGTTAATGACCTGAG	S72494
vacA-3*	1506 ←	GCCGATATGCAAAATGAGCCGC	S72494
cagA-1*	691 →	GATAACAGGCAAGCTTTTGAGGG	X70039
cagA-2 [‡]	864 →	GAAATTTGGGGATCAGCGTTACC	X70039
cagA-3*	1041 ←	TCCTGCAAAAAGATTGTTGGCAGA	X70039

(→) sense; (←) antisense direction of PCR primers and DNA hybridisation probes.

*PCR amplification primer.

[‡]DNA hybridisation probe used in Southern blot analysis of PCR amplified products.

[‡]DNA sequencing primer.

[§]Positions according to Gray *et al.* [25].

^{||}Positions according to referred accession numbers.

sham MP X-ray films at low temperature (−70°C) for 3–6 days. The *ureA*, *vacA* and *cagA* PCR-amplified products were analysed by agarose gel electrophoresis and Southern blot analysis, followed by hybridisation with *ureA*-2, *vacA*-2 and *cagA*-2 5' end-labelled hybridisation probes as described above. Hybridisations were performed as described earlier, except that post-hybridisation washing was in 2 × SSC/SDS 0.1%.

DNA sequencing

16S rDNA PCR amplified products were sequenced directly with an Fmol Cycle Sequencing kit (SDS Promega). The *Helicobacter*-specific primer HPV9 and the broad range primer pJB-1 were used in DNA sequence analysis. Cycle parameters were as follows: denaturation at 94°C for 20 s, annealing at 60°C for 20 s and extension at 70°C for 30 s (30 cycles). Exposed X-ray films were subsequently analysed with a computerised film scanner (SciScan 5000, Base-scanner software, United States Biochemical, Amer-sham). Comparative DNA sequence analysis with DNA databanks (EMBL and GeneBank) was performed.

Results

Identification of *Helicobacter* by PCR based on 16S rDNA sequences

Bacterial DNA was detected in *c.* 5 mg of gastric biopsy specimens. One antral and one corpus biopsy specimen from each individual were used in broad-range 16S rDNA PCR analysis. The results (Table 3) are presented at an individual level, not focusing on whether colonisation occurred in the antrum, corpus or both. In all biopsy specimens examined, a single 16S rDNA PCR-amplified product of *c.* 450 bp was detected on the ethidium bromide-stained agarose gel (data not shown). Thus, eubacteria of unknown identity were demonstrated in all 22 individuals (both gastritis and normal control groups). To verify the presence of *Helicobacter* in the biopsy specimens, PCR-amplified products were analysed by Southern blot analysis with a *Helicobacter*-specific 5' end-labelled probe, HPV4 (Table 2) which revealed the presence of *Helicobacter* in 17 of 22 biopsy specimens (12 from the gastritis group, the exception was No. 418, and five from the normal control group, Nos. 344, 436, 450, 484 and 492).

All results were obtained from at least two experi-ments on the same biopsy specimen, yielding identical results except in two cases (biopsy specimens from subjects 454 and 456).

Contamination controls were run in parallel. All controls were negative, indicating reliable DNA extraction and PCR amplification procedures.

Table 3. 16S rDNA sequence analysis and *vacA*, *cagA* and *ureA* hybridisation of PCR products

Parameter	<i>H. pylori</i> gastritis (subject nos.)														Histologically normal controls (subject nos.)							
	418	430	422	410	437	453	457	467	468	479	483	485	493	344	380	436	450	451	454	456	484	492
<i>Helicobacter</i> rod or cocci*	R	C	R	R	R	R	R	R	RC	R	R	R	R	—	—	C	—	—	—	C	—	—
16S rDNA sequencing†	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>vacA</i> hybridisation‡	—	+	+	—	+	—	—	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—
<i>cagA</i> hybridisation‡	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>ureA</i> hybridisation‡	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—	—	—	+	+

* *Helicobacter* rod-shape, R; coccoid, C; (—) no bacteria present as determined histologically.
† *Helicobacter*-specific 16S rDNA PCR with sequence analysis of the amplified products. Positive (+), and negative (—) DNA sequencing result.
‡ Hybridisation of PCR amplified products with 5'-end labelled hybridisation probes, as indicated. Positive (+), and negative (—) hybridisation result.

Partial 16S rDNA sequence analysis of DNA isolates

To assess possible DNA sequence diversity among different *Helicobacter* strains, 16S rDNA broad-range PCR-amplified products were sequenced in both directions. DNA sequence analysis turned out to be more sensitive than the Southern blot analysis and revealed the presence of *Helicobacter* in 20 of 22 subjects (Table 3). The partial 16S rDNA sequences from 14 subjects were identical to *H. pylori* type strain CCUG 17874^T (Fig. 1). However, six sequences deviated from that of the type strain in either of the variable regions V3 and V4, or both. Partial 16S rDNA sequences from subject nos. 422, 457 and 467 with gastritis were identical, showing two base shifts in the variable region V3 at locations 1030 and 1037, respectively (Fig. 1a). The 16S rDNA sequence from subject no. 479 (gastritis) showed the same variations in region V3 as the above-mentioned subjects, whereas subject no. 410 (gastritis) showed a base shift at location 1008. The 16S rDNA sequence from subject no. 453 (gastritis) was identical with the type strain in the variable region V3, whereas the 16S rDNA sequence deviated from the type strain in the variable region V4 at position 1136 (Fig. 1b). The 16S rDNA sequence from subject no. 479 (gastritis) showed two base shifts in positions 1151 and 1154, respectively (Fig. 1b). All other strains were identical with the type strain in this region.

The deviating strains were all found in biopsy samples from subjects with antral gastritis or pancreatitis. Only *H. pylori*-identical strains were identified in the *Helicobacter*-positive normal control group.

Characterisation of virulence factor genotype

The screening of *Helicobacter*-positive samples for *ureA*, *vacA* and *cagA* genotypes (identical to *H. pylori* type strain) revealed that these genotypes were more frequent in subjects with *H. pylori* gastritis than in the normal controls (Table 3). Biopsy samples from individuals with gastritis harboured *ureA*⁺/*cagA*⁺ *Helicobacter* strains. In this group, all individuals, except nos. 410, 418, 453 and 457, displayed a *H. pylori* type strain-identical *vacA*⁺ genotype. Among subjects in the normal control group shown to be *Helicobacter*-positive, the *ureA*⁺/*cagA*⁺ genotype was found in nos. 344, 436, 450, 484 and 492. Subjects 454 and 456 displayed only a *cagA*⁺ genotype. The type strain-identical *vacA* genotype was not identified in biopsy samples from individuals in the normal control group. The virulence genes *cagA* and *ureA* demonstrated in these normal controls support the results of 16S rDNA analysis, which makes the possibility of a false positive result due to contamination with PCR products unlikely.

The primer combinations used here were also tested on isolated human genomic DNA. No visible PCR

amplification products were detected on ethidium bromide-stained gels, demonstrating the bacterial DNA specificity of the primer combinations used (data not shown).

Comparison between 16S rDNA analysis, histology, rapid urease test and serology

The results of serology, histological examinations and rapid urease tests were in good agreement (Table 1). Histological examination of one subject with atrophic corpus gastritis (no. 430) revealed the absence of colonising rod-shaped *H. pylori*, but undefined coccoid cells were recognised; 16S rDNA analysis demonstrated the presence of *Helicobacter*. In the normal control group, two subjects (nos. 436 and 456) harboured undefined coccoid cells, which were confirmed as *Helicobacter* by 16S rDNA analysis. Among the nine histologically defined normal controls, 16S rDNA analysis revealed seven *Helicobacter*-positive cases (Table 3). In the gastritis group, 11 of 13 subjects colonised by *Helicobacter* were urease-positive. Subjects 418 and 430 were urease-negative, but 16S rDNA analysis revealed *Helicobacter*. 16S rDNA analysis and serology were in agreement in the gastritis group except in one case (no. 418) which was serum negative. Among the normal controls, serology detected one *Helicobacter*-positive subject (no. 454), whereas 16S rDNA analysis revealed *Helicobacter* in seven cases.

Discussion

This study isolated total DNA from gastric biopsy specimens from 22 asymptomatic individuals and verified the presence of nucleotide sequences corresponding to the genus *Helicobacter* in 20 of them by 16S rDNA, *vacA*, *cagA* and *ureA* gene analysis. It is believed generally that only *Helicobacter* colonise the stomach. However, the broad-range 16S rDNA PCR combined with Southern blot analysis of the PCR-amplified products showed that other bacterial species may be present in these specimens. It can be speculated that this is due to contamination with bacteria in swallowed saliva or respiratory secretions; however, other explanations are also possible. A recent study revealed that the commercially available Taq DNA polymerases contain minute amounts of DNA [28]. As a highly sensitive PCR assay (30 cycles, see ref. 28) was used in the present study, the possibility that part of the non-*Helicobacter*-specific DNA may be due to contaminating DNA in the commercial Taq DNA polymerase used cannot be excluded. Another explanation might be that other bacteria colonise the gastric mucosa, as Kabir and co-workers showed in a recent study with a gnotobiotic murine model [29]. *Helicobacter* DNA was also found in seven of nine individuals without gastritis (characterised as *H. pylori*-negative by urease test, histology and serology). This discrepancy may be due either to the high

A		V3			
		1000			1030
	<i>H. pylori</i> CCUG 17874 ^T	<u>TGAGAGAATC</u>	<u>CGCTAGAAAT</u>	<u>AGTGGAGTGT</u>	<u>CTGGCTTGCC</u> AG
a	Identical to type strain ¹	-----	-----	-----	-----
	410	-----	T-----	-----	-----
	422	-----	-----	-----	--A-----T--
	453	-----	-----	-----	-----
	457	-----	-----	-----	--A-----T--
	467	-----	-----	-----	--A-----T--
	479	-----	-----	-----	--A-----T--
b	<i>H. pylori</i> MC903	-----	-----	-----	-----T--
	<i>H. pylori</i> MC937	-----	-----	-----	-----
c	<i>H. rappini</i> ATCC 43879	---T-----	-----G--	-----*	-----
	<i>H. cinaedi</i> CCUG 18818 ^T	---T-----	-----G--	-----*	-----
	<i>H. fennelliae</i> CCUG 18820 ^T	---T-----	T-----G--	--C-----*	-----
	<i>H. heilmannii</i> isolate 2	---AG-----	T-----	--C-----	--A-----T--
	<i>H. acinonyx</i> CCUG 29263 ^T	---AN-----	T-----	-----	--A-----T--
	<i>H. nemestrinae</i> ATCC 49396 ^T	-----	-----	-----	-----A-T -A
B		V4			
		1120			1150
	<i>H. pylori</i> CCUG 17874 ^T	<u>TTTCTTAGTT</u>	<u>GCTAACAGGT</u>	<u>CATGCTGAGA</u>	<u>ACTCTAAGGA</u> TA
a	Identical to type strain ¹	-----	-----	-----	-----
	410	-----	-----	-----	-----
	422	-----	-----	-----	-----
	453	-----	-----	T-----	-----
	457	-----	-----	-----	-----
	467	-----	-----	-----	-----
	479	-----	-----	-----	-----G--A--
b	<i>H. pylori</i> MC903	-----	-----	-----	-----
	<i>H. pylori</i> MC937	-----	-----	-----	-----
	<i>H. pylori</i> MC123	-----	-----	T-----	-----
c	<i>H. rappini</i> ATCC 43879	G-C--N----	----G--C-	-G*-----C	-----G-
	<i>H. cinaedi</i> CCUG 18818 ^T	G-C-----	----G--C-	-G*-----C	-----G-
	<i>H. fennelliae</i> CCUG 18820 ^T	G-C-----	-T--G--T-	-G*-----C	-----G-
	<i>H. heilmannii</i> isolate 2	-----	-----	G-----C	T-----A- G-
	<i>H. acinonyx</i> CCUG 29263 ^T	-----	-----	--N-----	-----G-
	<i>H. nemestrinae</i> ATCC 49396 ^T	-----	-----*	-----	-----

Fig. 1. Partial 16S rDNA sequences from variable regions V3 (A) and V4 (B), respectively (underlined): a, from 20 biopsy samples that were identical to the type strain¹ including subject nos. 344, 418, 430, 436, 437, 450, 454, 456, 468, 483, 484, 485, 492 and 493; b, from *H. pylori* – isolates as previously published [26]; c, from related *Helicobacter* species known to colonise the gastro-intestinal tract of primates [27]. The 16S rDNA sequence from *H. heilmannii* isolate 2 was retrieved from Genebank/EMBL, accession number L10080. (–) denotes identical sequence to type strain, (*) denotes deletions.

sensitivity of the broad-range 16S rRNA gene PCR combined with DNA sequence analysis, or to contamination of biopsy samples. Contamination by means of transfer of bacteria with forceps or the gastroscope

from one patient to another seems unlikely (see disinfection procedures). Instead, the present data indicate that minute amounts of *H. pylori* DNA are nearly always present in gastric biopsy samples.

Provided that a significant fraction of *H. pylori*-infected individuals do not develop gastritis or circulating antibodies and that all six histologically examined biopsy samples (three corpus and three antrum biopsy samples from each subject) in each of seven normal controls (total of 42 biopsies) were taken in non-colonised areas (morphologically defined), a minor patchy colonisation pattern of *H. pylori* could have contributed to the discrepancies observed. The small size of gastric biopsy specimens, when only minor colonisation exists, may result in colonised and non-colonised biopsy specimens [16]. Unlike bacterial culture methods, most PCR techniques do not allow quantitative analysis; therefore, multiple analysis of the individual biopsy samples may help to overcome this problem. An advantage of PCR-based detection of *Helicobacter* is that non-culturable *Helicobacter* strains, either rod-shaped or coccoid forms can be detected by PCR amplification techniques. This fact became obvious in the analysis of normal controls, where it was found that seven of nine subjects harboured *Helicobacter* 'species'. Similar results have been reported by Hoshina and co-workers [13] and Lage and co-workers [17]. These investigators detected *H. pylori* and related helicobacters by means of DNA-based PCR in culture-, histology- and rapid urease test-negative biopsy specimens. However, in the study by Peek *et al.* [14], reverse-transcribed rather than DNA-based PCR was used. Peek *et al.* [14] did not compare 16S rDNA and *ureA* genotypes (DNA-based PCR) and the phenotypes (RT-PCR), which makes it difficult to evaluate their RT-PCR negative results. Low transcriptional activity, short half-life and degradation of bacterial mRNA and built-in errors in the RT-PCR technique [30] may explain the failure of Peek *et al.* [14] to detect all *H. pylori*-positive biopsy samples by RT-PCR.

The *cagA* genotype, which is associated with peptic ulcer disease, was found in biopsy samples from almost all subjects, including those without gastritis, which would render them potential candidates for ulcer development [5]. However, ulcer disease is generally not observed in the absence of gastritis. Previously, *cagA* genotypes have been reported to be present in *c.* 80% of clinical isolates [3], which is different from the results in the present study. On the other hand, the latter results agree with a study by Michlke and co-workers who reported that in patients from Korea and Houston *cagA* appeared to be present in a high proportion of *H. pylori* isolates (98.3% and 88%, respectively) [31]. The *cagA*⁺ result in an otherwise *Helicobacter*-negative normal control (no. 380) is difficult to explain. It is unlikely that this was caused by contamination with PCR product from another reaction. The same result has been obtained repeatedly.

The *vacA* genotype in this study is correlated with the *H. pylori* type strain CCUG 17874^T [32]. The PCR

primers used were designed to cover the mid-region of the *vacA* gene where deletions are observed in non-cytotoxin (tox⁻) and type strain-like cytotoxin-producing strains, respectively [33]. The hybridisation probe detected *vacA*⁺ type strain-identical *vacA* genotypes. All *vacA*⁺ isolates were identified among the subjects with *H. pylori* gastritis. In the normal control group all individuals harbouring *H. pylori* were *vacA*⁻. The possibility that these *H. pylori* strains are less pathogenic than the *H. pylori* type strain ATCC 43504 cannot be excluded.

The *ureA* genotype was expected to be present in all *Helicobacter*-positive strains. However, the study was unable to detect the *ureA* gene in two histologically normal controls, which were positive for *H. pylori* by 16S rDNA analysis and *cagA*⁺ (nos. 454 and 456). Tsuda and co-workers [34] showed that a urease-negative *H. pylori* mutant (rod-shaped) was unable to colonise the antrum of nude mouse mucosa. In contrast, Shen and co-workers [35] recently described the isolation of a new, urease-negative *Helicobacter* (*H. rodentium*). The possibility that a similar *Helicobacter* species was present among the human normal controls in the present study has not been ruled out. Although clinical isolates of *H. pylori* are generally urease positive, spontaneously arising urease-negative strains of *H. pylori* have been reported [36, 37]. Based on these findings, the urease-negative *H. pylori* in the normal control group cannot simply be treated as an artefact.

Partial 16S rDNA analysis revealed that six of 22 individuals harboured *Helicobacter* strains that differed from the *H. pylori* type strain in the variable regions V3 and V4 [25]. Thus, based on their different 16S rDNA sequences, two groups of *Helicobacter* could be detected in individuals with asymptomatic *H. pylori* gastritis – the type strain-identical group and a non-identical *H. pylori* group, respectively. These findings might indicate that different strains are associated with different types of gastritis, or inflammatory response, or both; but the clinical significance of 16S rDNA sequence variation remains unresolved. Admittedly, the present study only sequenced *c.* 250 bases of the whole 16S rDNA gene, covering two variable regions. However, initially PCR products were sequenced in duplicate yielding the same DNA sequence variations and, therefore, it is our opinion that the reported base variations are not the result of PCR artifacts. Moreover, these results are in agreement with previous findings where similar DNA sequence variations in the 16S rRNA gene from *Helicobacter* have been reported [26]. Comparison of the variants identified by us and others [26] with related *Helicobacter* species, known to colonise the human gastrointestinal tract, demonstrates an obvious relationship to the genus *Helicobacter* (Fig. 1). The taxonomy of these *H. pylori*-like strains may be a matter of debate. Subdivision of the species *H. pylori*

into subspecies, based on biotypes, pathotypes or serotypes for taxonomic as well as clinical reasons has been suggested recently [38]. If such *H. pylori* pathotypes exist, it may help to explain the results obtained from *H. pylori* isolates found in the normal control subjects of this study (see above). 16S rDNA sequences may not always be sufficient to allow taxonomic distinction between species or subspecies [39]. However, the partial 16S rDNA sequence data combined with the *ureA*, *cagA* and *vacA* patterns do support *H. pylori* subdivision [38].

The highly sensitive PCR-based molecular typing methods described here detect *H. pylori* in human gastritis biopsy samples. The PCR-based results are in good agreement with the results of morphology and serology. In contrast, different results were obtained with these methods in the analysis of human gastric biopsy samples from morphologically and serologically defined *H. pylori*-negative normal controls. Therefore, it may be argued that the overall application of molecular typing methods to identify *H. pylori* is not always appropriate, and thus may not replace commonly used typing and detection methods for patients with gastritis. On the other hand, these molecular typing methods are sensitive and discriminating enough to detect *Helicobacter* in human tissues from other areas of the gastrointestinal tract.

This study was supported by the Molecular Biology Program, LMÖ, University Hospital, Linköping, Sweden and grants from the Swedish National Cancer Association and the Swedish Medical Research Council. A. T. was supported by a Faculty Doctoral Fellowship from the Faculty of Health Sciences, University of Linköping, Sweden.

References

- McGowan CC, Cover TL, Blaser MJ. *Helicobacter pylori* and gastric acid: biological and therapeutic implications. *Gastroenterology* 1996; **110**: 926–938.
- Dooley CP, Cohen H, Fitzgibbons PL *et al.* Prevalence of *Helicobacter pylori* infection and histologic gastritis in asymptomatic persons. *N Engl J Med* 1989; **321**: 1562–1566.
- Xiang Z, Censini S, Bayeli PF *et al.* Analysis of expression of CagA and VacA virulence factors in 43 strains of *Helicobacter pylori* reveals that clinical isolates can be divided into two major types and that CagA is not necessary for expression of the vacuolating cytotoxin. *Infect Immun* 1995; **63**: 94–98.
- Cover TL, Tummuru MKR, Cao P, Thompson SA, Blaser MJ. Divergence of genetic sequences for the vacuolating cytotoxin among *Helicobacter pylori* strains. *J Biol Chem* 1994; **269**: 10566–10573.
- Atherton JC, Peek RM, Tham KT, Cover TL, Blaser MJ. Clinical and pathological importance of heterogeneity in vacA, the vacuolating cytotoxin gene of *Helicobacter pylori*. *Gastroenterology* 1997; **112**: 92–99.
- Georgopoulos SD, Mentis AF, Spiliadis CA *et al.* *Helicobacter pylori* infection in spouses of patients with duodenal ulcers and comparison of ribosomal RNA gene patterns. *Gut* 1996; **39**: 634–638.
- Nwokolo CU, Bickley J, Attard AR, Owen RJ, Costas M, Fraser IA. Evidence of clonal variants of *Helicobacter pylori* in three generations of duodenal ulcer disease family. *Gut* 1992; **33**: 1323–1327.
- van der Ende A, Rauws EAJ, Feller M, Mulder CJJ, Tytgat GNJ, Dankert J. Heterogenous *Helicobacter pylori* isolates from members of a family with a history of peptic ulcer disease. *Gastroenterology* 1996; **111**: 638–647.
- Relman DA, Schmidt TM, MacDermott RP, Falkow S. Identification of the uncultured bacillus of Whipple's disease. *N Engl J Med* 1992; **327**: 293–301.
- Monstein H-J, Kihlström E, Tiveljung A. Detection and identification of bacteria using in-house broad range 16S rDNA PCR amplification and genus-specific DNA hybridization probes, located within variable regions of 16S rRNA genes. *APMIS* 1996; **104**: 451–458.
- Tiveljung A, Backström J, Forsum U, Monstein H-J. Broad-range PCR amplification and DNA sequence analysis reveals variable motifs in 16S rRNA genes of *Mobiluncus* species. *APMIS* 1995; **103**: 755–763.
- Tiveljung A, Forsum U, Monstein HJ. Classification of the genus *Mobiluncus* based on comparative partial 16S rRNA gene analysis. *Int J Syst Bacteriol* 1996; **46**: 332–336.
- Hoshina S, Kahn SM, Jiang W *et al.* Direct detection and amplification of *Helicobacter pylori* ribosomal 16S gene segments from gastric endoscopic biopsies. *Diagn Microbiol Infect Dis* 1990; **13**: 473–479.
- Peek RM, Miller GG, Tham KT *et al.* Detection of *Helicobacter pylori* gene expression in human gastric mucosa. *J Clin Microbiol* 1995; **33**: 28–32.
- van Zwet AA, Thijs JC, Kooistra-Smid AMD, Schirm J, Snijder JAM. Sensitivity of culture compared with that of polymerase chain reaction for detection of *Helicobacter pylori* from antral gastric biopsy samples. *J Clin Microbiol* 1993; **31**: 1918–1920.
- Furuta T, Kaneko E, Suzuki M, Arai H, Futami H. Quantitative study of *Helicobacter pylori* in gastric mucus by competitive PCR using synthetic DNA fragments. *J Clin Microbiol* 1996; **34**: 2421–2425.
- Lage AP, Godfroid E, Fauconnier A *et al.* Diagnosis of *Helicobacter pylori* infection by PCR: comparison with other invasive techniques and detection of cagA gene in gastric biopsy specimens. *J Clin Microbiol* 1995; **33**: 2752–2756.
- Akopyanz N, Bukanov NO, Westblom TU, Kresovich S, Berg DE. DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. *Nucleic Acids Res* 1992; **20**: 5137–5142.
- Kansau I, Raymong J, Bingen E *et al.* Genotyping of *Helicobacter pylori* isolates by sequencing of PCR products and comparison with the RAPD technique. *Res Microbiol* 1996; **147**: 661–669.
- Lopez CR, Owen RJ, Desai M. Differentiation between isolates of *Helicobacter pylori* by PCR-RFLP analysis of urease A and B genes and comparison with ribosomal RNA gene patterns. *FEMS Microbiol Lett* 1993; **110**: 37–43.
- Petersson F, Jönsson K-Å, Franzén L, Borch K. Prevalence of gastritis and *Helicobacter pylori* infection in a randomly selected cohort of the general population aged 35–85 years in Sweden. Abstracts, Nordic Pathology Conference 1996; Kalmar, Sweden.
- Price AB. The Sydney System: Histological division. *J Gastroenterol Hepatol* 1991; **6**: 209–222.
- Dixon MF, Genta RM, Yardley JH, Correa P, and the participants in the International Workshop on the Histopathology of Gastritis, Houston 1994. Classification and grading of gastritis. The updated Sydney System. *Am J Surg Pathol* 1996; **20**: 1161–1181.
- Ma J-Y, Borch K, Sjöstrand SE, Janzon L, Mårdh S. Positive correlation between H,K-Adenosine triphosphatase autoantibodies and *Helicobacter pylori* antibodies in patients with pernicious anemia. *Scand J Gastroenterol* 1994; **29**: 961–965.
- Gray MW, Sankoff D, Cedergren RJ. On the evolutionary descent of organisms and organelles: a global phylogeny based on a highly conserved structural core in small subunit ribosomal RNA. *Nucleic Acids Res* 1984; **12**: 5837–5852.
- Eckloff BW, Podzorski RP, Kline BC, Cockerill FR. A comparison of 16S ribosomal DNA sequences from five isolates of *Helicobacter pylori*. *Int J Syst Bacteriol* 1994; **44**: 320–323.
- Cover TL, Blaser MJ. *Helicobacter pylori*: a bacterial cause of gastritis, peptic ulcer disease, and gastritis. *ASM News* 1995; **61**: 21–26.
- Hilali F, Saulnier P, Chachaty E, Andreumont A. Decontamination of polymerase chain reaction reagents for detection of low concentrations of 16S rRNA genes. *Mol Biotechnol* 1997; **7**: 207–216.
- Kabir AMA, Aiba Y, Takagi A, Kamiya S, Miwa T, Koga Y. Prevention of *Helicobacter pylori* infection by lactobacilli in a

- gnotobiotic murine model. *Gut* 1997; **41**: 49–55.
30. Sellner LN, Coelen RJ, Mackenzie JS. Reverse transcriptase inhibits Taq polymerase activity. *Nucleic Acids Res* 1992; **20**: 1487–1490.
  31. Miehke S, Kibler K, Kim JG *et al.* Allelic variation in the *cagA* gene of *Helicobacter pylori* obtained from Korea compared to the United States. *Am J Gastroenterol* 1996; **91**: 1322–1325.
  32. Telford JL, Ghiara P, Dell'Orco M *et al.* Gene structure of the *Helicobacter pylori* cytotoxin and evidence of its key role in gastric disease. *J Exp Med* 1994; **179**: 1653–1658.
  33. Atherton JC, Cao P, Peek RM, Tummuru MKR, Blaser MJ, Cover TL. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific *vacA* types with cytotoxin production and peptic ulceration. *J Biol Chem* 1995; **270**: 17771–17777.
  34. Tsuda M, Karita M, Morshed MG, Okita K, Nakazawa T. A urease-negative mutant of *Helicobacter pylori* constructed by allelic exchange mutagenesis lacks the ability to colonize the nude mouse stomach. *Infect Immun* 1994; **62**: 3586–3589.
  35. Shen Z, Fox JG, Dewhirst FE *et al.* *Helicobacter rodentium* sp. nov., a urease-negative *Helicobacter* species isolated from laboratory mice. *Int J Syst Bacteriol* 1997; **47**: 627–634.
  36. McLaren A, Cox DM, Snowden MA. Isolation and characterization of urease-negative variants of *Helicobacter pylori*. *Rev Esp Enferm Apar Dig* 1990; **78**: 29.
  37. Pérez-Pérez GI, Olivares AZ, Cover TL, Blaser MJ. Characteristics of *Helicobacter pylori* variants selected for urease deficiency. *Infect Immun* 1992; **60**: 3658–3663.
  38. Blaser MJ. Not all *Helicobacter pylori* strains are created equal: should all be eliminated? *Lancet* 1997; **349**: 1020–1022.
  39. Fox GE, Wisotzkey JD, Jurtshuk P. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int J Syst Bacteriol* 1992; **42**: 166–170.