

Rapid and specific detection of *Helicobacter pylori* macrolide resistance in gastric tissue by fluorescent in situ hybridisation

K Trebesius, K Panthel, S Strobel, K Vogt, G Faller, T Kirchner, M Kist, J Heesemann, R Haas

Abstract

Background—The development of macrolide resistance in *Helicobacter pylori* is considered an essential reason for failure of antibiotic eradication therapies. The predominant mechanism of resistance to macrolides, particularly clarithromycin, is based on three defined mutations within 23S rRNA, resulting in decreased binding of the antibiotic to the bacterial ribosome.

Aim—To develop an rRNA based whole cell hybridisation method to detect *Helicobacter* species in situ within gastric tissue, simultaneously with its clarithromycin resistance genotype.

Methods—A set of fluorescent labelled oligonucleotide probes was developed, binding either to *H pylori* 16S rRNA or 23S rRNA sequences containing specific point mutations responsible for clarithromycin resistance. After hybridisation and stringent washing procedures, labelling of intact single bacteria was monitored by fluorescence microscopy. The new approach was compared with PCR based assays, histology, and microbiological culture.

Results—In comparison with the phenotypic resistance measurement by E test, the genotypic clarithromycin resistance correlated perfectly (100%) for 35 *H pylori* isolates analysed. In a set of gastric biopsy specimens (27) *H pylori* infection was confirmed by histology (17/27) and correctly detected by whole cell hybridisation. Five clarithromycin resistant strains were identified in gastric tissue specimens directly. Furthermore, non-cultivable coccoid forms of *H pylori* were easily detectable by whole cell hybridisation.

Conclusions—Whole cell hybridisation of rRNA holds great promise for cultivation independent, reliable, and rapid (three hours) genotypic determination of clarithromycin resistance in *H pylori*. Compared with PCR techniques it is independent of nucleic acid preparations, not prone to inhibition, and allows semi-quantitative visualisation of the bacteria within intact tissue samples.

(Gut 2000;46:608-614)

Keywords: *Helicobacter pylori*; macrolide resistance; clarithromycin; in situ hybridisation; genotypic resistance

Helicobacter pylori is a bacterial pathogen related to a number of gastrointestinal disorders, including chronic gastritis, peptic ulceration, and mucosa associated lymphoid tissue (MALT) lymphoma.^{1,2} Numerous treatment regimens have been suggested to eradicate these small, slightly curved rods from the stomach mucosa. The most recent recommendation is triple therapy, including a proton pump inhibitor and either amoxicillin and clarithromycin, or metronidazole and clarithromycin.^{3,4} However, application of these antibiotics for the treatment of peptic ulcer and other diseases has led to the development of resistance to *H pylori*. The prevalence rates for developed countries are 11-70% for metronidazole resistance⁵ and up to 15% for clarithromycin resistance;⁶ only single cases of resistance to amoxicillin have been reported recently.⁷

Phenotypic resistance testing in vitro is usually not available until 48-96 hours after inoculation of agar plates. Determination of resistance based on genotype is, however, rapid and reliable. Resistance to clarithromycin in clinical *H pylori* isolates is caused predominantly by distinct point mutations within the peptidyl transferase centre of 23S rRNA.^{8,9} The most prevalent point mutations found in clinical isolates were A2143G and A2144G whereas transversion A2143C rarely occurs.^{8,10,11} Only a single clarithromycin resistant isolate with an A-to-G transition in positions 2116 and 2142 has been reported by Hulten *et al.*¹² Several different PCR based approaches for detection of the different point mutations have been developed which use amplification of a portion of domain V of 23S rRNA. The resulting amplicons were further analysed by the restriction enzymes *BsaI* and *MboII* or *BbsI* that specifically detect transitions in positions 2143 and 2144, respectively, but not transversion in position 2144.^{8,13,14} In contrast, application of a PCR-oligonucleotide ligation assay¹⁰ and a hybridisation based approach¹¹ detects all three mutations within this particular region.

Our study reports the successful application of a genotype based method for determination of clarithromycin resistance in *H pylori* by fluorescent in situ hybridisation.¹⁵ We applied

Abbreviations used in this paper: MIC, minimal inhibitory concentration; rRNA, ribosomal RNA; H-E stain, haematoxylin-eosin stain; MALT, mucosa associated lymphoid tissue; FLUOS, 5(6)-carboxy-fluorescein-N-hydroxysuccinimide ester.

Max von Pettenkofer
Institute for Hygiene
and Medical
Microbiology, Ludwig
Maximilians
University Munich,
Germany
K Trebesius
K Panthel
J Heesemann
R Haas

Institute for Medical
Microbiology and
Hygiene, University
Freiburg, Germany
S Strobel
M Kist

Department of
Microbiology, Hospital
Virchow and Charite,
Humboldt University
of Berlin, Germany
K Vogt

Institute of Pathology,
University of
Erlangen-Nürnberg,
Germany
G Faller
T Kirchner

Correspondence to:
Rainer Haas, Max von
Pettenkofer Institute for
Hygiene and Medical
Microbiology, Pettenkoferstr.
9a, D-80336 Munich,
Germany. Email:
haas@m3401.mpk.med.
uni-muenchen.de

Accepted for publication
25 November 1999

rRNA targeted fluorescent oligonucleotide probes for in situ identification of *H. pylori* and simultaneous determination of clarithromycin resistance within three hours. Compared with PCR based approaches, this technique does not require extensive nucleic acid preparation, is not prone to inhibition, and allows detection of *H. pylori* within intact tissue sections.

Materials and methods

BACTERIAL STRAINS AND GROWTH CONDITIONS

For *H. pylori* the type strain was obtained from the American Type Culture Collection (ATCC) whereas all other *H. pylori* strains were isolated from human gastric samples. The other reference strains used in this study were obtained from different strain collections, as specified in table 1. The *Helicobacter* strains, *Campylobacter jejuni*, and *Campylobacter coli* were grown on GC agar plates (Difco) supplemented with horse serum (8%), vancomycin (10 mg/l), trimethoprim (5 mg/l), and nystatin (1 mg/l) (serum plates), and incubated for 2–3 days in a microaerobic atmosphere (85% N₂, 10% CO₂, 5% O₂) at 37°C. *Wolinella succinogenes* and *C. rectus* were grown anaerobically as recommended by the suppliers (further details in table 1). Several colonies were taken from the plates, suspended in phosphate buffered saline (PBS) to an A₅₅₀ of 0.1, and fixed with 3% paraformaldehyde, as previously described.¹⁵ The *H. pylori* isolates used in this study were collected in 1996–1998 from our own institutions. For susceptibility testing and determination of the minimal inhibitory concentration (MIC), material from a pure culture plate was transferred with a sterile cotton swab into a 0.9% NaCl solution and adjusted to an optical density according to McFarland standard 1. This solution was flooded over Iso-Sensitest agar plates (OXOID), supplemented with 10% (v/v) horse blood (GIBCO). After drying of the plates, one E test strip (AB Biodisk, Solna, Sweden) was placed on each plate. The plates were incubated under micro-

aerophile atmosphere (5–10% O₂) at 37°C. MIC values were read after two days of incubation. As a quality control the reference strains *H. pylori* CCUG 38770, CCUG 38771, and CCUG 38772 with documented resistance against clarithromycin and metronidazole¹⁶ were used.

PREPARATION OF TISSUE SECTIONS

Human gastric biopsy specimens from the antrum or body mucosa of patients with upper abdominal complaints were taken during diagnostic endoscopy. To guarantee performance of the test, fixation of the biopsies should immediately follow sampling. Biopsies were fixed in 10% freshly prepared buffered formalin solution (incubation time in formalin should not exceed 48 hours), paraffin embedded and cut into 4 µm sections according to routine protocols. Gastritis was histologically diagnosed in haematoxylin-eosin (H-E) stains according to the updated Sydney system,¹⁷ and *H. pylori* and *H. heilmannii* infections were determined histologically using the Warthin-Starry stain. Eight of the samples positive for *H. pylori*, as identified by histology, were also analysed using cultivation techniques (including all five specimens with clarithromycin resistant *H. pylori*). Culture was performed as described above. *H. pylori* was identified by colony morphology (small translucent colonies) and a positive urease, catalase, and oxidase test.

PROBE DESIGN

A specific probe for *H. pylori* was developed using the ARB program package (Strunk O, Ludwig W. A microbiologist's sequence database tools; public domain software available at <http://www.biol.chemie.tu-muenchen.de/>). Approximately 10 000 complete or almost complete 16S rRNA sequences are contained in this database. The specificity of the developed probe Hpy-1 was confirmed by a gapped BLAST search.¹⁸ Oligonucleotide probes ClaR1, ClaR2, and ClaR3 were designed to

Table 1 Different bacterial strains and gastric biopsy samples analysed by fluorescent in situ hybridization

| Species | Source | Hpy-1 | ClaR1 ^a | ClaR2 ^b | ClaR3 ^c | ClaWT ^a | Eub ^d |
|---|----------------------------|-------|--------------------|--------------------|--------------------|--------------------|------------------|
| <i>H. pylori</i> (35) ^e | ATCC 43504+ 34 clin. isol. | 35 | 6 | 12 | 2 | 15 | 35 |
| <i>H. cinaedi</i> (1) | LMG ^g 13991 | 0 | 0 | 0 | 0 | ND | 1 |
| <i>H. bilis</i> (1) | ATCC 51630 | 0 | 0 | 0 | 0 | ND | 1 |
| <i>H. felis</i> (1) | ATCC ^f 49179 | 0 | 0 | 0 | 0 | ND | 1 |
| <i>H. femeliae</i> (1) | LMG 7546 | 0 | 0 | 0 | 0 | ND | 1 |
| <i>H. pullorum</i> (1) | NCTC ⁱ 12824 | 0 | 0 | 0 | 0 | ND | 1 |
| <i>H. mustelae</i> (1) | ATCC 43772 | 0 | 0 | 0 | 0 | ND | 1 |
| <i>C. jejuni</i> (1) | ATCC 33560 | 0 | 0 | 0 | 0 | ND | 1 |
| <i>C. rectus</i> (1) | DSM ^h 3266 | 0 | 0 | 0 | 0 | ND | 1 |
| <i>W. succinogenes</i> (1) | ATCC 29543 | 0 | 0 | 0 | 0 | ND | 1 |
| <i>P. vulgaris</i> (1) | ATCC 13315 | 0 | 0 | 0 | 0 | ND | 1 |
| <i>S. mutans</i> (1) | DSM ^h 20523 | 0 | 0 | 0 | 0 | ND | 1 |
| Gastric biopsy specimens (27) | | 17 | 4 | 1 | 0 | 13 ^k | 17 |
| Gastric biopsy specimen with <i>H. heilmannii</i> (2) | | 0 | 0 | 0 | 0 | 0 | 2 |

^aMIC for clarithromycin, as tested by the E test, >256 mg/l.

^bMIC for clarithromycin, as tested by the E test, 16–96 mg/l.

^cMIC for clarithromycin, as tested by the E test, >256 mg/l.

^dEub, universal bacterial 16S rRNA probe.¹⁹

^eTotal number in parentheses.

^fATCC, American Type Culture Collection (<http://www.atcc.org/>).

^gLMG, Laboratorium voor Mikrobiologie, Universiteit Gent, Belgium (<http://www.belspo.be/bccm/>).

^hDSM, Deutsche Stammsammlung für Mikroorganismen und Zellkulturen, Germany (<http://www.dsmz.de/>).

^kNumber of detected ClaR1, ClaR2, ClaR3, and ClaWT isolates within gastric samples exceeds total number of *H. pylori* positive biopsy specimens as one biopsy specimen contains a mixture of a WT and a ClaR1 isolate.

^lND, not determined.

Table 2 Sequences of the different oligonucleotide probes used in the present study

| Name | Sequence (5'-3') | Target (rDNA position ^a) | Specificity |
|-------------------|-----------------------|--------------------------------------|-------------------------------|
| Hpy-1 | CACACCTGACTGACTATCCCG | 16S (585–605) | <i>H. pylori</i> |
| Eub ¹⁹ | GCTGCCTCCCGT | 16S (338–349) | Bacteria |
| ClaR1 | CGGGGTCTTCCCGTCTT | 23S (2136–2152) | A2143G (Cla ^R) |
| ClaR2 | CGGGGTCTTCCCGTCTT | 23S (2136–2152) | A2144G (Cla ^R) |
| ClaR3 | CGGGGTCTTCCCGTCTT | 23S (2136–2152) | A2143C (Cla ^R) |
| ClaWT | CGGGGTCTTCCCGTCTT | 23S (2136–2152) | wild type (Cla ^R) |

^aNumbers for 16S rRNA sequence are according to *E. coli* numbering, 23S rRNA sequences refer to *H. pylori* numbers; 23S rDNA mutations leading to clarithromycin resistance are printed in bold and underlined.

detect the most prevalent 23S rRNA mutations responsible for clarithromycin resistance. Probe Eub,¹⁹ targeted to a 16S rRNA position almost universally present in bacteria, was used as a positive control. Hybridisation of this probe to a reference strain assures that oligonucleotides can readily penetrate cell boundaries of these particular bacterial cells and that these cells contain enough rRNA to be detected by in situ hybridisation. The probes, and their respective sequences and locations within the rRNA operons are summarised in table 2.

IN SITU HYBRIDISATION

Fluorescent oligonucleotide probes were ordered from Metabion, Munich, Germany. Probes were labelled with 5(6)-carboxy-fluorescein-N-hydroxysuccinimide ester (FLUOS; green fluorescence) or Cy3 (red fluorescence). In situ hybridisation was performed according to a protocol of Amann *et al.*¹⁵ Fixed bacterial strains were spotted on Teflon coated six well glass slides (Paul Marienfeld KG, Bad Mergentheim, Germany), air dried, and dehydrated in 50%, 80%, or 96% ethanol (three minutes each). Each well containing fixed cells was covered with 10 µl of hybridisation buffer (0.9 M NaCl; 0.02 M Tris/HCl, pH 8.0; 0.01% SDS) containing 30% formamide and 5 ng/µl of each probe. For glass slides carrying deparaffinised tissue sections, 50 µl of the same hybridisation buffer was added and coverslips were used to minimise

evaporation. Hybridisation was performed at 46°C for 90 minutes in a humid chamber and stringent washing at 48°C in a buffer containing 0.112 M NaCl, 20 mM Tris/HCl (pH 8.0) and 0.01% SDS. Hybridised samples were rinsed quickly with PBS, mounted in Citifluor (Citifluor Ltd, London, UK) and examined with the epifluorescence microscope Leica DMRBE (Leica, Heerbrugg, Switzerland) equipped with filters I3 for green fluorescence and N2.1 for red fluorescence. Photomicrographs were obtained by the confocal laser scanning microscope TCS NT by scanning one section with eight accumulations using Leica software.

CONFIRMATION OF CLARITHROMYCIN RESISTANCE TYPE

A 23S rDNA portion of all clarithromycin resistant *H. pylori* strains used in this study was amplified by PCR, as previously described.⁸ The hybridisations were performed blindly with no knowledge of the phenotypic resistance data. Point mutations A2143G and A2144G were detected by a restriction enzyme digest with *Mbo*II or *Bsa*I, respectively, according to Versalovic *et al.*⁸ For transversion A2143C, sequencing of the respective rDNA portion was performed. The internal 23S rRNA oligonucleotide primer 19 (Versalovic and colleagues⁸) was used for the Taq cycle DyeDeoxy terminator method, combined with an ABI PRISM 373A automatic sequencer (PE Applied Biosystems, Weiterstadt, Germany).

Results

COMPUTER ASSISTED PROBE DESIGN BASED ON rRNA SEQUENCE COMPARISON

Based on a comparative sequence analysis of nearly 10 000 bacterial 16S rRNA sequences contained in the ARB database, the oligonucleotide probe Hpy-1 was synthesised for specific detection of *H. pylori* by the whole cell hybridisation method. The 16S rRNA target sequence for this probe was found to be identical in all 10

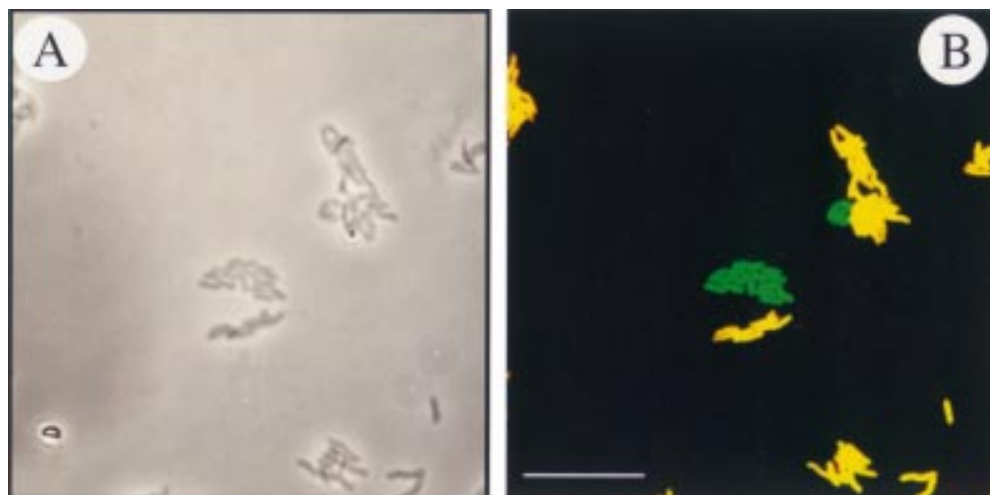


Figure 1 Specificity testing of oligonucleotide probe Hpy-1 for discrimination of *H. pylori* from related helicobacters. (A) A mixture of *H. pylori* and *H. mustelae*, as seen by phase contrast microscopy. (B) Detection of the bacterial mixture with two probes, a *H. pylori* specific probe Hpy-1-Cy3 (red) and a bacterial specific probe Eub-FLUOS (green). Both helicobacters bound the bacterial probe (green) but *H. pylori* bound Hpy-1-Cy3 (red) in addition, resulting in the yellow appearance of *H. pylori* (mixed colour). Bar represents 10 µm.

H. pylori strains available in the current rRNA databases and in *Helicobacter nemestrinae*, but in no other species. Probes ClaR1, ClaR2, and ClaR3 were designed and checked by the probe match tool to specifically bind to the 23S rRNA peptidyl transferase centre of *H. pylori* in a region covering the single point mutations responsible for clarithromycin resistance A2143G (ClaR1), A2144G (ClaR2), and A2143C (ClaR3) (see materials and methods).

SPECIFICITY EVALUATION OF PROBE Hpy-1 IN A WHOLE CELL HYBRIDISATION APPROACH

Probe Hpy-1 labelled with the fluorescent dye Cy3 (Hpy-1-Cy3) was tested for its specificity in binding to its target sequence using a set of independent *H. pylori* isolates grown on serum plates (table 1). All isolates (35/35) bound to Hpy-1 compared with none of the nine further species from the ϵ subclass of *Proteobacteria* (table 1, fig 1). The specificity of the probe was further analysed by hybridising *Lactobacillus lactis*, *Streptococcus mutans*, and *Proteus vulgaris* species which are sometimes recovered from the human stomach, but no binding of the probe was observed (data not shown). Thus we conclude that whole bacterial cells of *H. pylori*

hybridise to Hpy-1 and that the probe is specific and discriminates between closely related bacterial species that may be present in human gastric samples under the conditions used in our assay.

DETERMINATION OF MACROLIDE RESISTANCE IN *H. PYLORI* BY DETECTION OF POINT MUTATIONS

Next, probes ClaR1, ClaR2, and ClaR3 were evaluated for specific binding to respective target organisms. As a control, probe ClaWT was designed, which binds to the same rRNA region as the ClaR1–3 probes but hybridises to the wild type (WT) 23S rRNA sequence, as present in clarithromycin sensitive phenotypes. In total, 35 *H. pylori* isolates were analysed using probes ClaR1–3, ClaWT, and Hpy-1 (fig 2A, B). Twenty isolates were found to be clarithromycin resistant and 15 sensitive by E test.

There was 100% correlation between all three methods of measurement of resistance for all strains. Probes ClaR1, ClaR2, and ClaR3 hybridised only when the corresponding mutations were found by restriction analysis of corresponding PCR fragments or sequencing. Clarithromycin sensitive strains hybridised only with the ClaWT probe. However, all

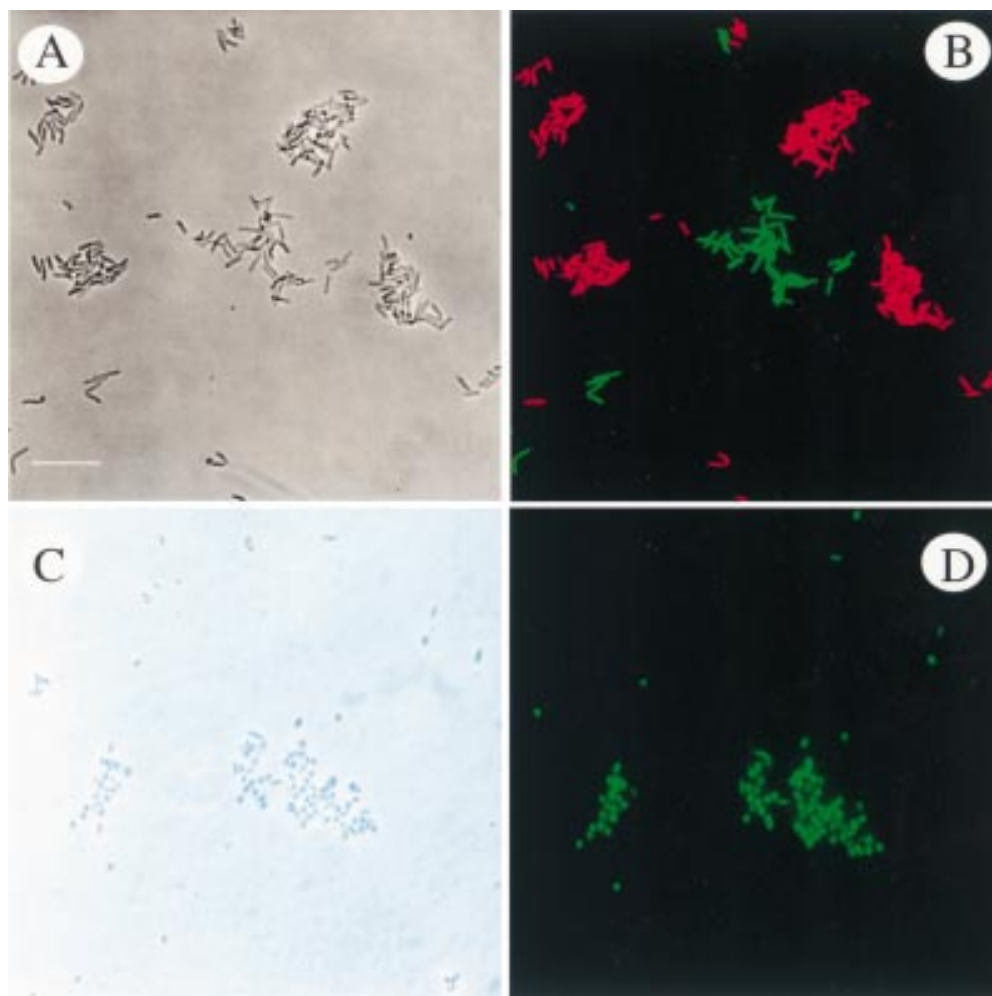


Figure 2 Specific detection of clarithromycin sensitive and resistant *H. pylori* isolates. (A) Phase contrast microscopy of a mixture of clarithromycin sensitive and resistant *H. pylori* isolates. (B) The same microscopic field after hybridisation with probes ClaWT-FLUOS (green) and ClaR1-Cy3 (red). (C) Phase contrast micrograph showing coccoid forms of clarithromycin resistant *H. pylori*. (D) Whole cell hybridisation of coccoid forms with probe Hpy-1-FLUOS. Bar represents 10 µm.

H. pylori strains hybridised with the Hpy-1 probe. Six isolates harboured the point mutation A2143G, 12 isolates carried transition A2144G, and only two isolates were found with the transversion A2143C (table 1). Thus the hybridisation method not only identified the species *H. pylori* specifically but also discriminated between single point mutations causing macrolide resistance. Point mutations at position 2143 (A→G or A→C) are often correlated with a high MIC for clarithromycin (≥ 256 $\mu\text{g/ml}$) whereas the point mutation in position 2144 confers variable MIC values to the respective strains.⁸ Therefore, binding of ClaR1, ClaR2, or ClaR3 to different resistant strains also provides information on the clarithromycin MIC of a particular isolate.²⁰

DETECTION OF CLARITHROMYCIN RESISTANCE IN COCCOID FORMS OF *H. PYLORI* BY FLUORESCENT IN SITU HYBRIDISATION

H. pylori isolates can develop from vegetative forms into coccoid, non-cultivable forms in vitro and in vivo.²¹ There has been much debate in the past, but no definite proof, on whether or

not such non-cultivable forms can form vegetative forms again. In the latter case they could play an important role in transmission of *H. pylori* and in survival after antibiotic treatment of patients. We asked therefore if coccoid forms can be specifically detected by fluorescent in situ hybridisation?

A clarithromycin resistant *H. pylori* isolate (ClaR1 specific) was used to induce coccoid forms by inoculating the bacteria for one week in distilled water at 4°C. The bacteria were hybridised to probes Hpy-1-FLUOS, ClaR1-Cy3, and ClaR2-Cy3 and analysed by phase contrast and fluorescence microscopy. Coccoid *H. pylori* cells showed bright fluorescence after hybridisation with probes Hpy-1 and ClaR1 but no fluorescence signal was detectable with probe ClaR2, used as a negative control. This demonstrated the specificity of the hybridisation and excluded a direct non-specific binding of the fluorescent dye or the oligonucleotide to coccoid cells. These data indicate that coccoid cells apparently harbour a high amount of ribosomal RNA and show that a genotypic clarithromycin resistance determination is pos-

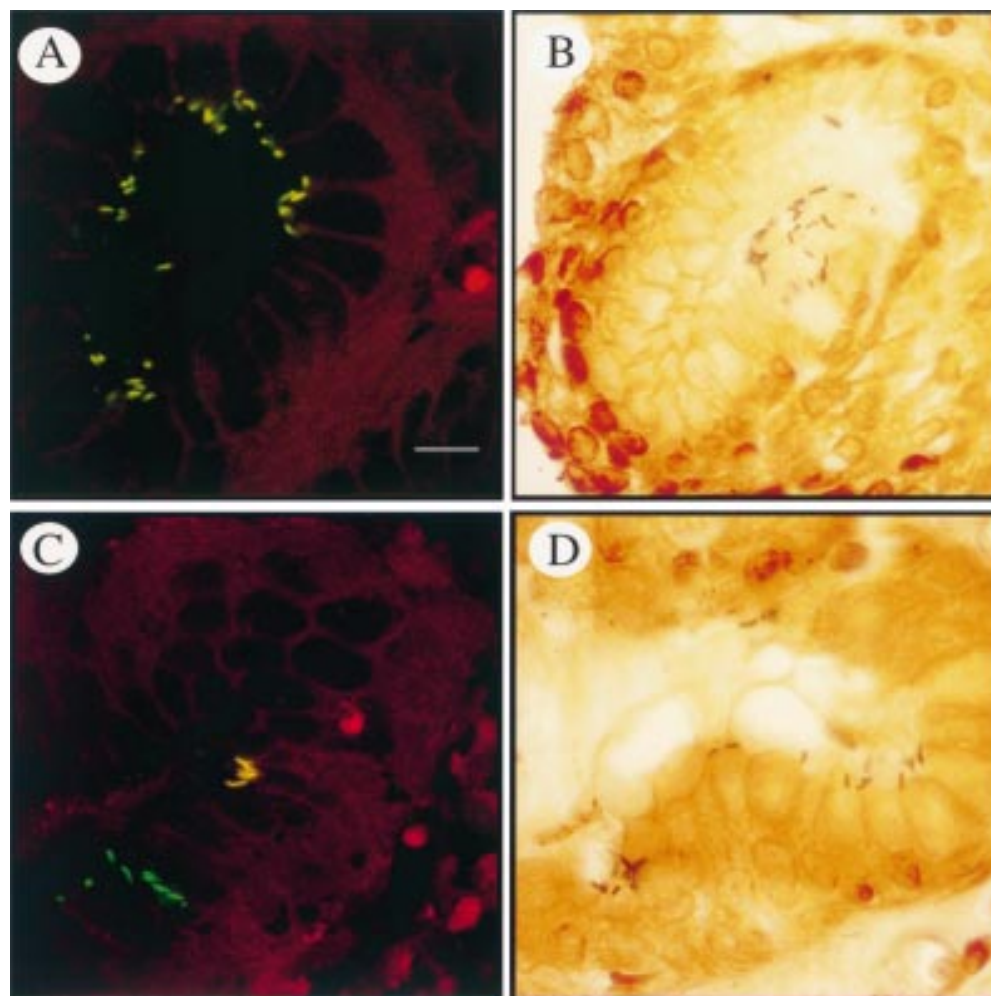


Figure 3 Specific detection of clarithromycin sensitive and resistant *H. pylori* isolates within gastric antrum sections of *H. pylori* infected patients. (Left) Detection of *H. pylori* and clarithromycin resistance by whole cell hybridisation with fluorescent probes Hpy-1-FLUOS and ClaR1-Cy3 simultaneously. (Right) Antrum sections from the same biopsy material (Warthin-Starry stain). (A) Clarithromycin resistant *H. pylori* are visible in yellow (mixed colour between green and red). (C) Mixed population of clarithromycin resistant (yellow) and sensitive (green) *H. pylori* in the same biopsy specimen of a patient. (B, D) Antrum sections showing *H. pylori* by the Warthin-Starry stain, which does not allow resistance determination.

sible in coccoid forms, although they cannot be cultured (fig 2C, D).

DETECTION OF CLARITHROMYCIN RESISTANT *H. PYLORI* IN TISSUE SECTIONS

The next challenging step was to identify *H. pylori* and its resistance genotype directly within gastric tissue sections. Therefore, formalin fixed tissue sections from gastric biopsy specimens of 27 patients were investigated by the detection strategy mentioned above. *H. pylori* were isolated from 17 tissue samples by microbiological culture and verified as *H. pylori* by routine diagnostics. The resistance phenotype was determined by E test.

All tissue sections which were culture positive for *H. pylori* (17/27) were diagnosed as *H. pylori* positive by histology using Warthin-Starry staining. In the same 17 sections, but not in the remaining 10 sections, *H. pylori* was detected by hybridisation of probe Hpy-1-FLUOS (table 1). Although a moderate background fluorescence was seen, spiral *H. pylori* were clearly visible as individual bacteria by whole cell hybridisation, as demonstrated for two samples (fig 3A, C). Thus the sensitivity and specificity of the assay was 100% compared with histology. Twelve *H. pylori* containing biopsy specimens did not hybridise to any of the ClaR probes and were identified as clarithromycin sensitive. The spiral shaped bacteria within four of the remaining biopsy specimens hybridised to probe ClaR1 and one bound to probe ClaR2 (table 1).

The biopsy specimen of one patient harboured two different *H. pylori* strains, one carrying a wild type sequence (hybridising to ClaWT) and one hybridising to probe ClaR1 (fig 3C, D). From this patient, only the resistant *H. pylori* was recovered by culture. An E test performed with the 17 isolated strains identified the same five isolates as clarithromycin resistant as found by whole cell hybridisation (table 1). Thus there was complete correlation between phenotypic and genotypic determination of clarithromycin resistance.

Discussion

The macrolide clarithromycin has been included in the most recent treatment regimens for *H. pylori* because it readily penetrates into the gastric mucosa and has a low MIC for *H. pylori*, even at low pH values.²² Unfortunately, application of this potent antimicrobial drug is associated with increasing numbers of resistant *H. pylori* strains. Contrary to metronidazole, another antibiotic highly effective against

H. pylori, resistance to clarithromycin is always correlated with a reduction in therapeutic efficiency.²³ We have reported the successful application of fluorescent in situ hybridisation for simultaneous detection of *H. pylori* and the respective 23S rRNA point mutations responsible for macrolide resistance.

Ribosomal RNA targeted in situ hybridisation has already been applied for the detection of *H. pylori* in gastric tissue samples²⁴ but to our knowledge ours is the first report on the successful application of this technique for the

detection of antibiotic resistance in bacteria. In the latter study, a long part of the *H. pylori* 16S rDNA was used as a hybridisation probe. Although strong hybridisation signals were obtained, the hybridisation protocol was tedious and probe penetration within deeper areas of tissue was limited by the size of the polynucleotide probe. Furthermore, non-specific binding of the probe to other *Helicobacter* species was observed.

Compared with other staining techniques in gastric samples (e.g. silver staining) our approach is highly specific for *H. pylori*. None of the other bacterial species hybridised to Hpy-1 whereas all 35 clinical *H. pylori* isolates in this study did. A probe mixture containing all ClaR probes labelled with Cy3, a FLUOS-labelled Hpy-1, and unlabelled probe ClaWT allowed simultaneous detection of *H. pylori* and the point mutations accounting for clarithromycin resistance within three hours. The correlation between phenotypic and genotypic methods for resistance determination was 100% for the strains and gastric samples we tested. However, a small number of clarithromycin resistant *H. pylori* strains have been reported which apparently carry different point mutations conferring clarithromycin resistance.¹⁴ These strains cannot be addressed by the present whole cell approach unless corresponding oligonucleotides are designed and tested.

Besides *H. pylori*, the only other *Helicobacter* species rarely found in the human gastric mucosa is *Helicobacter heilmannii*, a non-cultivable species associated with gastritis. The species-specific probe contained in our oligonucleotide mixture did not cross hybridise with this species or other *Helicobacter* species isolated from human samples.

Contrary to previously reported PCR based methods for the detection of clarithromycin resistance in *H. pylori*, our approach could be directly applied to formalin fixed tissue sections without extensive preparation of nucleic acid.²⁵ Another advantage of this technique compared with PCR based systems became obvious when mixed strains and tissue sections harbouring more than one *H. pylori* strain were examined. A mixed culture cannot be unequivocally differentiated from a strain carrying two different rRNA operons by restriction enzyme analysis or filter hybridisation; this can be done easily by whole cell hybridisation technology.

Transformation of spiral *H. pylori* to coccoid forms has been postulated to account for therapy failure, development of resistance, and recurrent infection with this gastric pathogen.²⁶ Although several studies reported low RNA content in such morphological forms, we have successfully hybridised "culture resistant" coccoid forms and found large amounts of ribosomal RNA. As long as there is no definite proof that these forms cannot revert to vegetative forms in vivo (e.g. after incomplete eradication therapy) they have to be regarded as potentially pathogenic, and fluorescent in situ hybridisation is a powerful tool to monitor the occurrence of such forms in the stomach mucosa in situ.

Although the number of strains and biopsy samples studied for this report are too small to draw definitive conclusions, this highly specific technique holds great promise as a rapid and reliable method for the detection of *H pylori* in human gastric tissue samples. Other possible sources to identify *H pylori* by whole cell hybridisation in the future include cryosections of gastric tissue or simple smears of gastric mucous or surface mucous cells, which would allow more rapid detection without the need to prepare sections. Pathogenic *Yersinia* species have already been successfully identified in stool and throat samples²⁷ and future studies will show if *H pylori* can be detected within these complex samples by fluorescent in situ hybridisation.

In conclusion, fluorescent in situ hybridisation has been proved as a reliable and rapid means of in situ detection of both *H pylori* and the point mutations responsible for clarithromycin resistance. As these promising results have been generated with a limited number of strains and samples, more studies using greater numbers of samples have already been launched to further validate the sensitivity and specificity of the method. This technique has some advantages over other competitive techniques, as outlined above. In the future it may be possible to replace the fluorescence based detection system with an enzyme coupled system which would allow a combination of specific detection with conventional histology staining methods. In our opinion, the whole cell hybridisation technology should be considered for routine application in the future as it is cost effective, easy to implement, and rapid.

This study was supported by a grant from Creatogen GmbH, Augsburg, Germany. The authors are grateful to Kristin Adler for excellent technical assistance.

- 1 Blaser MJ. Hypotheses on the pathogenesis and natural history of *Helicobacter pylori* induced inflammation. *Gastroenterology* 1992;102:720-7.
- 2 Wotherspoon AC, Dogliani C, Diss TC, et al. Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of *Helicobacter pylori*. *Lancet* 1993;342:575-7.
- 3 Anonymous. Current European concepts in the management of *Helicobacter pylori* infection. The Maastricht Consensus Report. European *Helicobacter Pylori* Study Group. *Gut* 1997;41:8-13.
- 4 Lind T, Veldhuyzen van Zanten S, Unge P, et al. Eradication of *Helicobacter pylori* using one-week triple therapies combining omeprazole with two antimicrobials: the MACH I study. *Helicobacter* 1996;1:138-44.
- 5 Dunn BE, Cohen H, Blaser MJ. *Helicobacter pylori*. *Clin Microbiol Rev* 1997;10:720-41.
- 6 Mégraud F. Antibiotic resistance in *Helicobacter pylori* infection. *Br Med Bull* 1998;54:207-16.
- 7 van Zwet AA, Vandenbroucke-Grauls CM, Thijs JC, et al. Stable amoxicillin resistance in *Helicobacter pylori*. *Lancet* 1998;352:1595.
- 8 Versalovic J, Shortridge D, Kibler K, et al. Mutations in 23S rRNA are associated with clarithromycin resistance in *Helicobacter pylori*. *Antimicrob Agents Chemother* 1996;40:477-80.
- 9 Debets-Ossenkopp YJ, Sparrius M, Kusters JG, et al. Mechanism of clarithromycin resistance in clinical isolates of *Helicobacter pylori*. *FEMS Microbiol Lett* 1996;142:37-42.
- 10 Stone GG, Shortridge D, Versalovic J, et al. A PCR-oligonucleotide ligation assay to determine the prevalence of 23S rRNA gene mutations in clarithromycin-resistant *Helicobacter pylori*. *Antimicrob Agents Chemother* 1997;41:712-14.
- 11 Pina M, Occhialini A, Monteiro L, et al. Detection of point mutations associated with resistance of *Helicobacter pylori* to clarithromycin by hybridization in liquid phase. *J Clin Microbiol* 1998;36:3285-90.
- 12 Hulten K, Gibreel A, Skold O, et al. Macrolide resistance in *Helicobacter pylori*: mechanism and stability in strains from clarithromycin-treated patients. *Antimicrob Agents Chemother* 1997;41:2550-3.
- 13 Szczebara F, Dhaenens L, Vincent P, et al. Evaluation of rapid molecular methods for detection of clarithromycin resistance in *Helicobacter pylori*. *Eur J Clin Microbiol Infect Dis* 1997;16:162-4.
- 14 Occhialini A, Urdaci M, Doucet-Populaire F, et al. Macrolide resistance in *Helicobacter pylori*: rapid detection of point mutations and assays of macrolide binding to ribosomes. *Antimicrob Agents Chemother* 1997;41:2724-8.
- 15 Amann RI, Krumholz L, Stahl DA. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J Bacteriol* 1990;172:762-70.
- 16 Glupczynski Y, Mégraud F, Andersen LP, et al. Antibiotic susceptibility of *H. pylori* in Europe in 1998: results of the third multicentre study. *Gut* 1999;45:A3.
- 17 Dixon MF, Genta RM, Yardley JH, et al. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol* 1996;20:1161-81.
- 18 Altschul SF, Madden TL, Schaffer AA, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997;25:3389-402.
- 19 Amann RI, Binder B, Olson RJ, et al. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* 1990;56:1919-25.
- 20 Garcia-Arata IM, Baquero F, De Rafael L, et al. Mutations in 23S rRNA in *Helicobacter pylori* conferring resistance to erythromycin do not always confer resistance to clarithromycin. *Antimicrob Agents Chemother* 1999;43:374-6.
- 21 Cole SP, Cirillo D, Kagnoff MF, et al. Coccoid and spiral *Helicobacter pylori* differ in their abilities to adhere to gastric epithelial cells and induce interleukin-8 secretion. *Infect Immun* 1997;65:843-6.
- 22 Mégraud F. Epidemiology and mechanism of antibiotic resistance in *Helicobacter pylori*. *Gastroenterology* 1998;115:1278-82.
- 23 Graham DY. Antibiotic resistance in *Helicobacter pylori*: implications for therapy. *Gastroenterology* 1998;115:1272-7.
- 24 Karttunen TJ, Genta RM, Yoffe B, et al. Detection of *Helicobacter pylori* in paraffin-embedded gastric biopsy specimens by *in situ* hybridization. *Am J Clin Pathol* 1996;106:305-11.
- 25 Marais A, Monteiro L, Occhialini A, et al. Direct detection of *Helicobacter pylori* resistance to macrolides by a polymerase chain reaction/DNA enzyme immunoassay in gastric biopsy specimens. *Gut* 1999;44:463-7.
- 26 Axon AT, Moayyedi P. Eradication of *Helicobacter pylori*: omeprazole in combination with antibiotics. *Scand J Gastroenterol Suppl* 1996;215:82-9.
- 27 Trebesius K, Harmsen D, Rakin A, et al. Development of rRNA-targeted PCR and *in situ* hybridization with fluorescently labelled oligonucleotides for detection of *Yersinia* species. *J Clin Microbiol* 1998;36:2557-64.