NOTES

Detection of *Helicobacter pylori* in Paraffin-Embedded and in Shock-Frozen Gastric Biopsy Samples by Fluorescent In Situ Hybridization

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Received 25 July 2002/Returned for modification 3 October 2002/Accepted 31 October 2002

We report on the successful application of fluorescent in situ hybridization for detection of *Helicobacter pylori* and determination of its clarithromycin susceptibility in formalin-fixed and paraffin-embedded gastric biopsy specimens that had been prepared for pathological examination. This method is useful when results from conventional culturing with antibiotic susceptibility testing are not available.

Clarithromycin resistance in *Helicobacter pylori* is based on single-base mutations within the peptidyltransferase-encoding region of the 23S rRNA gene (4, 7, 9). At the Max von Pettenkofer-Institute, we have established the fluorescent in situ hybridization (FISH) technique with rRNA-targeted fluorescence-labeled oligonucleotide probes specific for three described mutations in which the adenine residues at positions 2143 and 2144 are replaced by guanine (A2143G and A2144G) and cytosine (A2143C), respectively (8). Recently, we have demonstrated the application of FISH for (i) simultaneous detection of *H. pylori* and identification of the 23S rRNA point mutations responsible for macrolide resistance directly from shock-frozen gastric biopsy specimens (5) and (ii) rapid and accurate determination of genotypic clarithromycin resistance in cultured *H. pylori* isolates (6). FISH has been implemented at the Max von Pettenkofer-Institute as a work technique that is part of the daily routine and was found to be a more sensitive and reliable method than conventional culturing for detection of *H. pylori* in gastric biopsy specimens (5).

Ideally, antral biopsy specimens taken during diagnostic endoscopy from patients with upper abdominal complaints are sent to (i) a microbiology laboratory for culturing of *H. pylori* and subsequent susceptibility testing with all relevant antibiotic drugs and to (ii) a pathologist for histological diagnosis of gastritis according to the updated Sydney system and histological determination of *H. pylori* infection by adequate staining techniques such as those that use the Wharthin-Starry or modified Giemsa stain. The “gold standard” for accurate diagnosis of an *H. pylori* infection is either culturing of the pathogen and/or concordant positive results obtained by histology and the rapid urease test or the ¹³C-urea breath test (UBT), since

none of these diagnostic tests except bacterial culturing are 100% specific. The major advantage of FISH is the fact that the rRNA-targeted fluorescence-labeled oligonucleotide probes can be used for accurate determination of macrolide susceptibility, thus providing the clinician with important information with which to make a proper treatment recommendation. Clarithromycin is a key component of most triple therapies for eradication of *H. pylori* (2), and the resistance of the pathogen to this antibiotic is an important cause of treatment failure, particularly in children, who are reported to harbor *H. pylori* strains with a high rate of primary resistance to this macrolide (1, 3).

The aim of this retrospective study was to evaluate the sensitivity and specificity of FISH for *H. pylori* detection in formalin-fixed and paraffin-embedded gastric biopsy specimens in comparison to those determined from previously obtained results of this hybridization technique with shock-frozen biopsy specimens. At the University Children's Hospital (Ludwig Maximilians-Universität München), antral biopsy specimens obtained during upper endoscopy from patients with dyspeptic symptoms, abdominal pain, or peptic ulcer disease are routinely sent both to the Institute of Pathology for histological examination and to the microbiology laboratory for bacterial culturing and FISH with shock-frozen tissue. For this study, we investigated paraffin-embedded biopsy specimens from 100 randomly chosen patients of the Pediatric Department (age range, 0.7 to 29 years; median age, 10.4 years) that had been obtained during endoscopy 1 to 4 years earlier for histopathological examination. The definition of positivity for *H. pylori* infection was a positive culture result. For all positive patients, the results of at least two of three nonculture tests (UBT, rapid urease test, histology) were also positive. In contrast, the patient was considered not infected with *H. pylori* when culture, histology, UBT, and the rapid urease test gave concordant negative results. According to this definition, 46 patients were

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FIG. 1. Application of FISH for *H. pylori* detection in formalin-fixed and deparaffinized gastric biopsy specimens and in shock-frozen gastric biopsy specimens from 100 dyspeptic patients. UBT, ^{13}C -urease breath test; RUT, rapid urease test; Cla-S, clarithromycin sensitive; Cla-R, clarithromycin resistant.

not infected with the pathogen and 54 patients were infected with the pathogen (Fig. 1).

The pathologist provided seven 4- μm -thick sections from each formalin-fixed and paraffin-embedded biopsy specimen. The specimens were coded to ensure complete blinding of the microbiologist. Sections were spotted onto glass slides and fixed by incubation overnight at 55°C. On the next day, the tissue sections were deparaffinized as follows. The glass slides were dipped twice into hexane for 30 min each time and, subsequently, twice into sterile absolute ethanol for 30 min each time. After the slides were air dried at room temperature, FISH was performed as described previously (5, 6, 8). Briefly, oligonucleotide probe Hpy-1 (5'-CACACCTGACTGACTATCCCG-3'), targeted to a 16S rRNA position, was used to specifically identify *H. pylori* in gastric tissue sections, whereas probes ClaR1 (which targets A2143G; 5'-CGGGGTCTTCCC GTCTT-3'), ClaR2 (which targets A2144G; 5'-CGGGGTCT CTCCGTCTT-3'), and ClaR3 (which targets A2143C; 5'-CG GGGTCTTGCCGTCTT-3') were designed to detect the 23S rRNA point mutations responsible for the clarithromycin resistance of the pathogen. The probes used for this study were synthesized (Metabion, Munich, Germany) and 5' labeled with the fluorochrome Cy3 for probes ClaR1, ClaR2, and ClaR3 (which gives a red signal) or the fluorochrome fluorescein isothiocyanate for probe Hpy-1 (which gives a green signal). The tissue sections were overlaid with 50 μl of hybridization

buffer (0.9 M NaCl, 0.02 Tris-HCl [pH 8.0], 0.01% sodium dodecyl sulfate) containing 30% formamide and an oligonucleotide mixture (5 ng/ μl) consisting of probes ClaR1, ClaR2, ClaR3, and Hpy-1. Hybridization was carried out at 46°C for 90 min in a humid chamber. Then, the tissue sections underwent a stringent washing at 48°C in a buffer containing 0.112 M NaCl, 20 mM Tris-HCl (pH 8.0), and 0.01% sodium dodecyl sulfate. Subsequently, the samples were stained with 4',6-diamidino-2-phenylindole (DAPI), which binds to DNA and which thus helped to localize the microorganisms during microscopy.

As shown in Fig. 1, application of FISH to both shock-frozen and deparaffinized gastric biopsy specimens gave concordant negative results for specimens from all 46 patients negative for *H. pylori* infection (specificity, 100%). Among the specimens from 54 patients infected with *H. pylori*, FISH identified the pathogen in 50 shock-frozen biopsy specimens and 51 deparaffinized biopsy specimens (sensitivities, 93.1 and 94.7%, respectively). Regarding the detection of clarithromycin-sensitive and clarithromycin-resistant strains, the results were consistent for 47 of the 54 patients. Thirty-seven patients were infected with a clarithromycin-sensitive strain, 5 patients were infected with a clarithromycin-resistant strain, and 5 patients had mixed infections with a clarithromycin-sensitive strain and a clarithromycin-resistant strain, as determined by both FISH applications. For the remaining seven *H. pylori*-positive patients, three shock-frozen biopsy specimens were probed and were found to be positive for the pathogen, whereas no bacteria could be detected by FISH in the respective deparaffinized specimens. Vice versa, four deparaffinized biopsy specimens were positive for *H. pylori* by FISH, whereas the respective shock-frozen tissue sections were negative for the pathogen. Even extended examination of more than seven tissue sections from deparaffinized biopsy specimens with false-negative results gave no positive FISH results. Moreover, DAPI staining was not positive for bacteria in any of these sections. These false-negative results are probably due to the fact that *H. pylori* is not evenly distributed within the stomach (8) and that the tissue sections of the particular antral biopsy specimen indeed did not contain the bacterial pathogen.

In conclusion, application of FISH to deparaffinized gastric biopsy specimens up to 4 years old for detection of *H. pylori* and determination of its genotypic macrolide susceptibility is as sensitive as application of FISH to freshly prepared, shock-frozen biopsy specimens. This technique is very valuable in situations in which results from conventional culturing with antibiotic susceptibility testing are not available for clinical decision making.

This study was supported by Astra-Zeneca, Wedel, Germany, and the Stiftung Kindergesundheits. H.R. was supported by the AIDS-Stipendienprogramm from the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie of Germany.

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