original article

Rapid detection of clarithromycin-resistant *Helicobacter pylori* in patients with dyspepsia by fluorescent in situ hybridization (FISH) compared with the E-test

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BACKGROUND: Clarithromycin is the antibiotic of choice for treatment of *H. pylori*-related dyspepsia, but unfortunately, resistance to clarithromycin is not rare. Detection of resistant strains takes 2 to 4 days by conventional methods. In this report, we applied the FISH technique for rapid detection of *H. pylori* in biopsies of dyspeptic patients.

METHODS: Gastric biopsies from 50 patients suffering from dyspepsia were tested in this study. Part of each biopsy specimen was cultured and the remainder was fixed in liquid nitrogen. After mounting of frozen sections on microscopic slides, they were hybridized with oligonucleotide probes for detection of clarithromycin-resistant *H. pylori*. The slides were visualized under a fluorescent microscope. Susceptibility of cultured strains of *H. pylori* to clarithromycin was also determined by the E-test and the results were compared.

RESULTS: Twenty-five of 50 biopsy specimens examined by FISH were positive for *H. pylori*. FISH showed that 17 strains (68%) were susceptible to clarithromycin and 6 strains (24%) were resistant. Bacteria isolated following culture of 2 biopsy specimens had a mixture of both clarithromycin-susceptible and resistant strains (8%). There was no discrepancy between the E-test and FISH technique for detection of resistant strains of *H. pylori*.

CONCLUSION: FISH is a rapid technique for detection of *H. pylori* in clinical samples. Moreover, strains susceptible to clarithromycin can be detected quickly. Therefore, this method is suitable for determination of susceptibility of *H. pylori* to clarithromycin, especially when a quick decision is necessary for treating dyspeptic patients.

Helicobacter pylori has been recognized as one the most common chronic bacterial infections worldwide.[1] Isolation of this bacterium from the majority of patients with peptic ulceration, chronic gastritis or gastric lymphoma has demonstrated a strong relationship between presence of *H. pylori* and development of ulcers or dyspepsia.[2,3]

Recent reports have shown that reinfection with *H. pylori* is frequent, especially in children.[4] Eradication of *H. pylori* in patients is possible by triple-therapy, which includes application of a proton pump inhibitor (PPI) combined with two antibiotics (amoxicillin and clarithromycin or metronidazole and clarithromycin).[5,6] A triple regimen including raberprazole, amoxicillin and gatifloxacin has also been recommended for treatment of *H. pylori* infection with a highlt effective eradication rate.[7] However, the effect of antibiotics on patients living in different geographical area is slightly different. While for Japanese patients the combination of a PPI drug with amoxicillin and metronidazole is highly effective,[8] a report shows that a combination of new antibiotics such as erythromycin and rifamycin derivatives with new polycyclic compounds has a powerful antibacterial activity on *H. plyori*.[9] In addition, emergence of resistant strains of *H. pylori* is usual.[10,11,12] The resistance rates of *H. pylori* strains to metronidazole increased from 35.2% to 78.5% between 1987-1988 and 1990-1996, respectively, in Saudi Arabia, while the resistance to tetracycline pr amoxicillin was rare in Saudi Arabia and Bahrain.[13,14] Detection of the resistant strains by

conventional bacteriological methods takes at least 2 to 4 days and it may be lead to a delay in treatment, but FISH technique allows detection of clarithromycin-resistant strains of *H. pylori* within 2 to 4 hours.[15] In this study we applied fluorescent in situ hybridization (FISH) for determination of susceptibility or resistance of *H. pylori* to clarithromycin in the patients with dyspepsia, and compared the results to the E- test.

METHODS

Gastric biopsy specimens taken from the antrum and corpus of 50 dyspeptic patients were immediately transferred to our microbiology lab in a suitable transport medium (Portagerm pylori, Biomerieux, Marcy, I`Etoile, France). Part of each biopsy was used for culture and the other part was fixed on a microscopic slide.

Biopsies were fixed in Tissue-Tek compound (USA) and freezed in liquid nitrogen (-196°C).[16] Each tissue piece was cut into 4-µm sections by cryomicrotom (Leica GM3050, Germany) and put into electrostatic superfrost plus slide[15] (Menzel-Glaser, Germany). All probes used in our study have been previously described.[15,17] A specific oligonucleotide [Hpy probe labelled with fluorescein (FLOUS) dye] targeted to a 16S rRNA (TIB, Molbiol, Berlin, Germani) was used to identify *H. pylori*. Also, probes of ClaR1, ClaR2, ClaR3 (labeled with the fluorochromes Cy3) and ClaWT (Metabion, Munich, Germany) were used for detection of 23S rRNA point mutations responsible for clarithromycin resistance. Control slides were prepared[18,19,20,21] by using mutant (resistant) and wild (susceptible) strains of *H. pylori*. Also, Campylobacter jejuni was used as a negative control.

Ten and 40 µL of hybridization solution containing a mixture of 5 probes (5 ng/µL) for each one) was added to each well of the control and fixed gastric biopsy slides, respectively. After hybridization at 46°C for 90 minutes in a humid chamber and washing by buffer,[15,16] the slides were stained by DAPI (4`,6`-diamidino-2-phenylindol).[16] Hybridized samples were mounted by DAKO solution, covered with coverslips and examined under a fluorescent microscope equipped with a standard filter set.

The other part of the gastric biopsy specimen was homogenized and inoculated onto selective media of Colombia and Brucella agar.[15,22,23] Isolated colonies suspected to be *H. pylori* were identified by Gram stain, rapid urease test, catalase, oxidase[22,24,25] and susceptibility to cephalothin and nalidixic acid (30 µg disks).

Mueller-Hinton agar containing 5% sheep's blood and a clarithromycin tape (AB Biodisk,Solna, Sweden) was used for the E-test.[16,22] Twenty-four isolates of *H. pylori* recovered from 50 gastric biopsy specimens were examined for susceptibility to clarithromycin by the E-test. *H. pylori* isolates with a MIC (minimum inhibitory concentration) ≥ 2 µg/mL were considered resistant.[15,16,23] The results of *H. pylori* resistance in both the FISH and E-test methods were compared with each other.

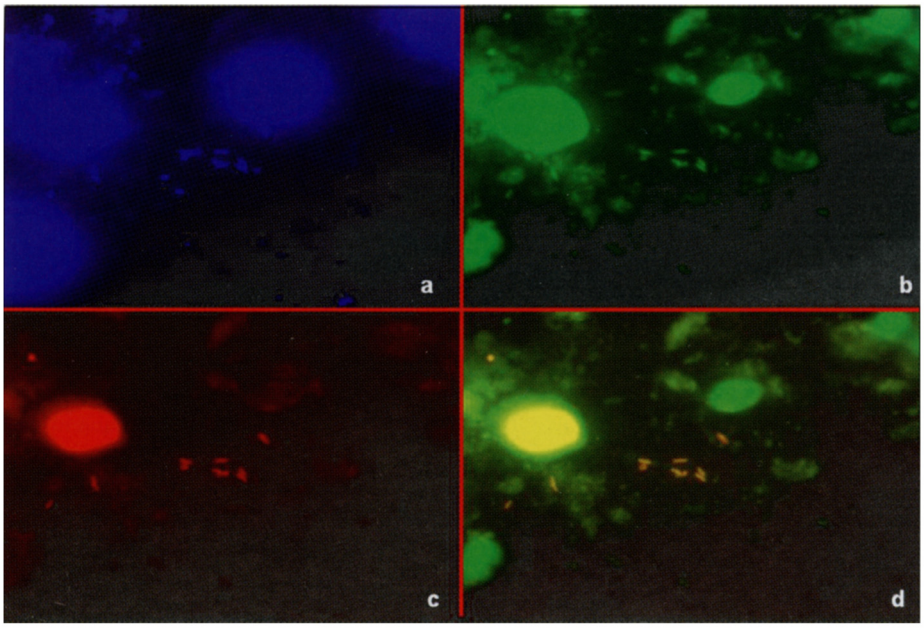

Figure 1. Clarithromycin-resistant *H. pylori* in gastric biopsy specimens. All four pictures show one similar microscopic field. (a) DAPI blue signal. (b) Green signal by Hpy-FLUOS hydridization. (c) Red signal by ClaR-Cy3 hydridization. (d) Yellow signal under mixed filter.

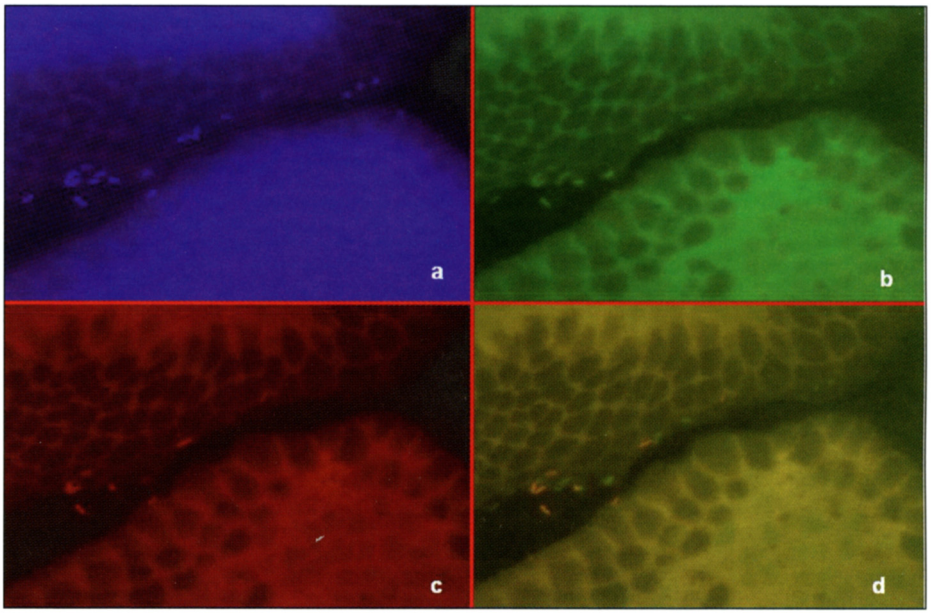

Figure 2. Resistant and sensitive strains of *H. pylori* to clarithromycin in gastric biopsy specimens. (a) DAPI blue color. (b) Green signals by hybridization of Hpy-FLUOS probe with both sensitive and/or resistant strains of *H. pylori*. (c) Red signal by hydridization of ClaR-Cy3 probe with only resistant *H. pylori*. (d) Yellow or orange signal is related to the sensitive strain.

RESULTS

Resistant (mutant) strains of *H. pylori* ClaR1, ClaR2 and ClaR3 were hybridized with the Cy3-labled probes ClaR1, ClaR2 and ClaR3, respectively. Also, these strains were hybridized with Hpy probe labelled with FLUOS dye (green). Therefore, resistant strains were visualized red under a red filter of a fluorescent microscope and emitted yellow or orange signals under a mixed filter. However, susceptible *H. pylori* strains (wild type) hybridized with both probes Hpy (green) and ClaWT (without fluorescent dye) were observed only with green signals (under both green and mixed filters). *Campylobacter jejuni* (as negative control) was not hybridized with probes Hpy and ClaR, and so was observed blue (Figures 1 and 2).

The examination of a total of 50 gastric biopsy specimens resulted in detection of 25 *H. pylori* strains, when the Hpy probe was used by the FISH technique. Also, FISH showed that 17 (68%) of these 25 strains were susceptible (hybridized with ClaWT probe), 6 (24%) were resistant (hybridized with ClaR1-3 probes) and 2 strains (8%) were a mixture of susceptible and resistant strains to clarithromycin. In this study, 5 isolates of *H. pylori* (21%) with an MIC more than 2 μg/mL were considered as resistant, while the MIC 0f 17 isolates (71%) was below 2 μg/mL by the E-test. The results obtained by both FISH and E- test were in full concordance with each other (Table 1). In this study, FISH technique was applied in 3 hours.

DISCUSSION

Despite effective treatment regimens containing tetracycline, metronidazole, and amoxicillin against *H. pylori*, the most suitable regimen contains clarithromycin and amoxicillin.[26] Relative resistance against gastric pH, suitable absorption when prescribed orally, the long half-life of the drug and low gastrointestinal side effects make clarithromycin a useful drug for eradication of *H. pylori* infections.[11,27] However, *H. pylori* is gradually getting resistant to clarithromycin. It is estimated that currently up to 15% to 20% of *H. pylori* isolates, especially from children, are resistant to clarithromycin.[11,28,29]

This study determined status of resistance or susceptibility of *H. pylori* to clarithromycin by the FISH method in a maximum of 3 hours, which it is very rapid compared with traditional methods (48-96 hours). Furthermore, by using 5 probes (Hpy, ClaR1-3 and ClaWT) the FISH technique could detect *H. pylori* and 23S rRNA point mutations within the peptidyltransferase region simultaneously. These mutations [adenine (A) at positions 2143 and 2144 replaced by guanine (G- A2143G, A2144G) or cytosine(C-A2143C] are known as causative agents of *H. pylori* resistance to clarithromycin in gastric biopsy specimens.[15,16]

The data obtained by the FISH technique in this study was in coordination with data obtained from the E- test (Table 1) because all susceptible or resistant isolates known by the E- test were the same as those seen directly in clinical specimens by the FISH method.

Table 1. Susceptibility or resistance results for *H. pylori* to clarithromycin by fluorescent in sity hdribization (FISH) and E-test methods, using a mixture of 5 probes for hybridization.

No. of isolates	FISH probes			E-test
	Hpy	ClaWT	ClaR (1-3)	
17 (68%)	Positive	Positive	Negative	Susceptible
5 (20%)	Positive	Negative	Positive	Resistance
1 (4%)	Positive	Positive	Negative	No growth
2 (8%)	Positive	Positive/negative	Positive/negative	Susceptible/resistant mixed infection*
Total				25 (100%)

*Mixed Infection, including 2 susceptible and resistant isolates to clarithromycin shown by FISH and MIC values < 2µg/mL (susceptible) and ≥ 2 µg/mL (resistant) by the E-test.

However, the E-test was performed on 24 *H. pylori* isolates because no growth was seen in one gastric biopsy specimen (Table 1). The reason for this difference is that coccoid forms of *H. pylori* are not culturable, but they are visible by the FISH test.[15] Perhaps the bacterium loses its viability during transport to the lab, so no growth will be seen on culture medium, while the FISH technique could detect it in the specimen.[16]

On the other hand, the FISH technique showed both susceptible and resistant strains of *H. pylori* in gastric biopsies, simultaneously, and this is important in view of successful treatment of the patients. Some reports have been shown that the FISH method could identify a low population of clarithromycin-resistant strains easier than the E-test.[16] The incidence of clarithromycin-resistant *H. pylori* has been reported as about 15%,[15,16] therefore, in 85% of cases, susceptible strains of this bacterium will be determined by the FISH method in a short time. In comparison with former methods, *H. pylori* resistance (or MIC) will be determined by the E-test for only a few isolates (recovered from specimens during 48 to 96 hours incubation), on the agar plate, after 2 days.

We conclude that FISH technique is a rapid technique for detection of *H. pylori*, and related clarithromycin-resistant strains. This technique is a suitable method to determine the susceptibility of *H. pylori* to clarithromycin, especially in situations in which the physician needs to decide quickly on treatment.

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