



Clinical role and importance of fluorescence *in situ* hybridization method in diagnosis of *H pylori* infection and determination of clarithromycin resistance in *H pylori* eradication therapy

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Abstract

H pylori is etiologically associated with gastritis, gastric and duodenal ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. Eradicating *H pylori* may convert rapidly the outcome of related diseases with the use of more accurate diagnostic molecular tests. Indeed some of the tests cannot give the evidence of current infection; *H pylori* can be detected by noninvasive and invasive methods, the latter requiring an endoscopy. Eradication failure is a big problem in *H pylori* infection. Recently, clarithromycin resistance in *H pylori* strains is increasing and eradication therapy of this bacterium is becoming more difficult. Molecular methods have frequently been applied besides phenotypic methods for susceptibility testing to detect clarithromycin resistance due to mutations in the 2143 and 2144 positions of 23S rRNA gene. Fluorescence *in situ* hybridization (FISH) method on paraffin embedded tissue is a rapid, accurate and cost-effective method for the detection of *H pylori* infection and to determine clarithromycin resistance within three hours according to the gold standards as a non-culture method. This method can also be applied to fresh biopsy samples and the isolated colonies from a culture of *H pylori*, detecting both the culturable bacillary forms and the coccoid forms of *H pylori*, besides the paraffin embedded tissue sections. This technique is helpful for determining the bacterial density and the results of treatment where clarithromycin has been widely used in populations to increase the efficacy of the treatment and to clarify the treatment failure *in vitro*.

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Key words: *H pylori*; Fluorescence *in situ* hybridization method; Clarithromycin resistance

INTRODUCTION

H pylori is etiologically associated with gastritis, gastric and duodenal ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma^[1-3]. The prevalence of *H pylori* infection is 70%-90% in developing countries and 25%-50% in developed countries^[2,4]. Person-to-person spread is the most probable mode of transmission. Fecal-oral and oral-oral transmission are also reported^[5]. *H pylori* has been classified as a class I carcinogen by International Agency for Cancer Research (IACR)^[6]. *H pylori* can be detected by noninvasive and invasive methods, the latter requiring an endoscopy. Noninvasive testing for *H pylori* can be done by measuring exhaled ¹³C urea breath test (UBT), by serology, by stool antigen tests, by stool PCR and by analyzing body materials such as saliva and urine^[2,7]. Invasive tests include endoscopy, with biopsy of the affected region followed by histopathologic examination of stained specimens to demonstrate the presence of the bacterium, rapid urease test, biopsy PCR and culture of the bacterium^[1]. UBT, histopathology and culture of the organism, although not easily and routinely performed, are considered the gold standard for the diagnosis of *H pylori* infection^[1,8].

Seven and fourteen days of triple therapy are recommended to eradicate *H pylori* according to Maastricht 2-2002 Consensus report. The triple therapy comprises a proton pump inhibitor in combination with two antibiotics, including amoxicillin, clarithromycin, or metronidazole^[9-11]. Clarithromycin is a key component of most treatment recommendations to eradicate *H pylori*^[12]. Resistance of *H pylori* to clarithromycin is regarded as a particular dilemma, since this drug is a part of both established therapy regimens. Thus, macrolide resistance is a frequent cause for failure of *H pylori* eradication therapy^[10]. In industrialized countries approximately 10% of the

H. pylori strains are clarithromycin-resistant. In developing countries, resistance rates to clarithromycin are higher, varying between 25% to 50%, while they are 5%-10% in the USA and as high as 10% in Europe^[10,13,14]. Recently, the clarithromycin resistance rates are reported 16.8% and 52%-56% in Turkey^[15-17]. In routine clinical laboratories the detection of clarithromycin resistance for *H. pylori* is mainly based on phenotypic methods performed after culture: agar diffusion for the E-test or the agar dilution method, which is preferred as a reference method. However, there are some disadvantages of these methods such as results are not available until 48-96 h after inoculation of the agar plates^[10,18]. Because *H. pylori* is a fastidious organism, there are also some problems in application of culture and antibiotic susceptibility testing. Therefore easy, cheap and practicable methods are required for the detection of *H. pylori* and determination of resistance, which is very important before the treatment if the resistance reaches 15%-20% in the area^[7,19]. When the bacterial culture cannot be used routinely, the patient should benefit from the determination of macrolide resistance using non-invasive genotype-based methods^[18].

Resistance of *H. pylori* to clarithromycin is mainly due to major point mutations within an adenine-to-guanine transition at positions A2142G, A2143G and A2144G and to an adenine-to-cytosine transversion at positions A2142C and A2143C, which are included in the peptidyltransferase-encoding region of the 23S rRNA^[9,11,20-23]. Mutations A2142G and A2143G are the most often observed, with the A2142C mutation being less common^[24]. Other point mutations A2115G, G2141A, and T2717C have also been reported, though they appear to be very rare^[18,25-27].

Detection of point mutations conferring resistance to clarithromycin for *H. pylori* by molecular methods may constitute a more reliable approach and is attracting more attention^[18]. Some molecular-based methods have been developed. PCR-based methods have been used to determine susceptibility to clarithromycin from biopsy specimens or cultured *H. pylori* strains and stool specimens^[7,28-30]. Fluorescence *in situ* hybridization (FISH) method for detection of *H. pylori* and determination of its genotypic macrolide susceptibility in gastric biopsy specimens is a cultivation independent, reliable, sensitive and specific method^[9,10,24]. Detection of *H. pylori* and clarithromycin resistance genotype simultaneously in gastric biopsy specimens by FISH method may be a good tool for research in the future for the drug resistance mechanisms and to search for the eradication failure in developing countries, such as clarithromycin and similar antibiotics^[23,31].

CLARITHROMYCIN ACTIVITY AND MECHANISM OF RESISTANCE

Clarithromycin activity

Clarithromycin is a bacteriostatic antibiotic, which belongs to a group of macrolides binding to peptidyl transferase loop of domain V of the 23S rRNA molecule. This binding interferes with protein elongation, and thus effectively blocks bacterial protein synthesis. The

antibacterial activity of clarithromycin is similar to that of other macrolides, but clarithromycin is better absorbed in the gastric mucus layer, more acid-stable, and therefore more effective against *H. pylori*^[13,26,32]. Resistance to clarithromycin is thought to develop when substitutions in one amino acid at or near this binding site on the ribosome prevent the drug from binding, thereby making it ineffective^[13].

Clarithromycin resistance

Resistance to clarithromycin in *H. pylori* is caused by point mutations in three adjacent 23S rRNA nucleotides, namely 2142, 2143 and 2144. In *H. pylori* these substitutions cause decreased affinity of the ribosomes for several macrolides, resulting in increased resistance. It can be induced by an adenine (A) to guanine (G) substitution at one of these positions or an adenine (A) to cytosine (C) substitution solely at position 2142. The A2142G and A2142C were significantly more frequently present in isolates with a higher minimal inhibitory concentration (MIC) for clarithromycin (> 64 mg/L), whereas the A2143G substitution was often found in isolates with a lower MIC (< 64 mg/L). Occasionally, other 23S rRNA mutations have also been reported for *H. pylori*; some of them are associated with high-level resistance, while others are associated with low-level resistance^[26,32]. Occasionally three described mutations, in which the adenine residues at positions 2143 and 2144 are replaced by guanine (A2143G and A2144G) or cytosine (A2143C), are localized within the peptidyl transferase region of the 23S rRNA gene^[9,11,22,31].

H. pylori contains two 23S rRNA genes and mutations are generally found in both copies, however, heterogeneity has been described. Heterogeneity still results in clarithromycin resistance, but it generally appears to be associated with lower resistance levels than in homogenic isolates. The higher prevalence of homogeneity over heterogeneity in *H. pylori* may reflect a high efficiency of DNA recombination. The mutation in one copy of the 23S rRNA may be easily transferred to the other 23S rRNA gene by efficient homologous DNA recombination under selective pressure, conferring higher levels of clarithromycin resistance^[11,22,24,26,31,32]. As expected, clarithromycin resistance coincides with resistance to other macrolides. The A2142G and A2142C mutations are linked to high-level cross-resistance to all macrolides, whereas the A2143G mutation gives rise to high-level resistance to erythromycin and intermediate-level resistance to clindamycin and streptogramin^[26,32].

DETECTION OF CLARITHROMYCIN RESISTANCE

Molecular tests

The association between point mutations in the 23S rRNA gene and macrolide resistance in *H. pylori* potentially provides a new approach for diagnosing macrolide resistant *H. pylori* strains^[33]. Numerous molecular-based methods are now available to assess clarithromycin in *H. pylori*, such as PCR-RFLP, PCR-OLA, PCR-DEIA, PCR-

LipA, PCR-PHFA, 3M-PCR, real-time PCR hybridization assay, FISH, FRET, DNA sequencing by conventional and real-time (pyrosequencing) techniques. Most assays are polymerase chain reaction (PCR)-based using different methods to study the amplicons. The PCR-based molecular techniques are quicker than microbiological susceptibility testing, and more importantly, they can be performed directly on gastric biopsies and gastric juice^[33].

Restriction fragment length polymorphism (RFLP) is a simple method based on the occurrence of restriction site within the amplicon. This assay allows for the detection of the previously mentioned 23S rRNA mutations using the restriction endonucleases, *Mbo*II (A2142G) *Bbs*I (A2142G), *Bsa*I (A2143G) and *Bce*AI (A2142C). As the PCR-RFLP was initially not able to detect the A2142C mutation, a 3'-mismatch reverse primer PCR method (3M-PCR) was developed^[23,25,27]. However, the mutations are identified by the absence of a band, which is a less preferable endpoint than a positive endpoint^[27].

Other methods, such as PCR-DNA enzyme immunoassay (DEIA), PCR oligonucleotide ligation assay (OLA), preferential homoduplex formation (PHFA) and PCR-line probe assay (LipA), include an additional hybridization step after the PCR. The PCR products were hybridized with labeled oligonucleotide probes under highly stringent conditions and hybrids were subsequently detected with specific antibodies or streptavidin-alkaline phosphatase^[13,18,22,27,34,35]. The PHFA has been applied to direct detection of *H pylori* and clarithromycin resistant mutants in gastric juice samples^[27]. The PHFA uses double labeled amplicons. Many of the assays are based on the principle of reverse hybridization with labeled probes for up to seven mutations and the wild type, immobilized either in microtitre wells (DEIA) or on nitrocellulose (LIPA). In these assays, PCR products are hybridized to the probes under highly stringent conditions and the resultant hybrids are detected colorimetrically. In the DEIA, the detection system is an enzyme linked immunoabsorbent assay with a labeled anti-double stranded DNA monoclonal antibody. Other more complex microtitre plate based systems such as the OLA use labeled capture and reporter probes^[35].

Recently, several real-time PCR hybridization assays have been developed. The real-time PCR technique, which is powerful advancement of the basic PCR method, is developed based on amplification of a fragment of the 23S rRNA gene of *H pylori* followed by the melting curve analysis by biprobes and hypoprobes^[18,36]. In these assays a 23S rDNA fragment is amplified in the presence of a fluorescent-labeled mutation and anchor probe. Biprobes are sequence-specific probes labeled with the fluorophore Cy5. When the probe hybridizes to the target sequence, Cy5 is excited by the energy transfer from SybrGreen I, resulting in an increase of emitted light^[29,36-38]. After completion of the PCR, the temperature is increased to determine the melting point of the mutation probe. The temperature at which the fluorescent signal drops indicates the point at which the mutation probe dissociates (melting point). When there are mismatches present in the target sequence, lower melting temperatures are obtained compared to the matched hybrid. This technique is simple and quick, and if applied directly to gastric tissue, results

can be obtained within 3 h^[13,27,29,34-38].

A technique named fluorescence resonance energy transfer (FRET) can be applied. In the first article in 1999, a DNA double strand specific fluorophor SYBR Green I and a second fluor dye Cy5 on a probe were used to test *H pylori* strains. This method was then applied to gastric biopsies^[26].

In contrast with all the above approaches, DNA sequencing provides the gold standard reference method for mutation detection although it is not technically feasible or cost effective for routine laboratory determination of *H pylori* resistance markers. Nevertheless, knowledge of nucleotide sequences has proved invaluable for validation of the various assays mentioned above, particularly where a resistant phenotype is not associated with any of the more common mutations^[35].

A recent development in rapid sequencing based on the principle of pyrosequencing, a real time DNA sequence analysis of short (25-30 bp) DNA stretches, has been applied to rapid identification of *H pylori*. Available data suggest this new technique can offer an accurate and rapid method for sequence analysis of PCR amplicons providing easily interpreted results within hours^[35].

Fluorescence in situ hybridization

In situ hybridization (ISH) uses a labeled probe to detect and localize specific RNA or DNA sequences in a tissue or on a chromosome. ISH relies on DNA's ability to re-anneal, or hybridize, with a complimentary strand when at the correct temperature. "*In situ*" means "in the original place" in Latin, so ISH involves a labeled nucleic acid probe hybridizing with a DNA or RNA sequence *in situ* (in the cells) so that the location of the sequence of interest can be detected in the cells, tissue, or chromosome. Like Northern and Southern blots, ISH indicates the presence of a particular RNA or DNA sequence, but ISH differs from blots in that the labeled probe reveals the actual location of the sequence in the cells. The probe can be either radioactively labeled and detected by autoradiography or fluorescently labeled (abbreviated FISH) and detected by immunocytochemistry. The specificity of the probe depends on the permeability of the cells, the type of probes, the labeling technique, and the hybridization conditions, so specificity of ISH can be adjusted according to the desired results^[39,40].

Fluorescence *in situ* hybridization (FISH) is to identify the presence of specific chromosomes or chromosomal regions through hybridization or fluorescence-labeled DNA probes to denatured chromosomal DNA^[41]. FISH uses fluorescent molecules to vividly paint genes on chromosomes. This technique is particularly useful for gene mapping and for identifying chromosomal abnormalities. FISH involves the preparation of short sequences of single stranded DNA, called probes, which are complementary to the DNA sequences that researchers wish to paint and examine. These probes hybridize, or bind, to the complementary DNA and, because they are labeled with fluorescent tags, allow researchers to see the location of those sequences of DNA^[41,42]. This technique allows the detection of whole bacteria in their natural habitat by fluorescence microscopy of prepared

specimens. Fluorescent signals indicate the presence of complementary chromosomal DNA; the absence of fluorescent signals indicates absence of complementary chromosomal DNA^[43]. Unlike most other techniques used to study chromosomes, which require that the cells be actively dividing, FISH can also be performed on non-dividing cells, making it a highly versatile procedure^[41,42,44].

Despite their small size, bacteria are accessible to the tools of cytology, such as immunofluorescence microscopy for localizing proteins in fixed cells with specific antibodies, fluorescence microscopy with the green fluorescent protein for localizing proteins in live cells, and FISH for localizing chromosomal regions and plasmids within cells^[45].

The bacterial FISH technology is based on the specific DNA-DNA hybridization of defined oligonucleotides with the abundant copies of ribosomal RNA of a bacterial species (16S rRNA, 23S rRNA). The oligonucleotides, which are labeled with fluorescent dyes, penetrate the bacterial cells and bind to their target sequence. This technique allows the detection of whole bacteria in their natural habitat by fluorescence microscopy of prepared specimens, i.e. the gastric mucosa of infected humans or from animal models^[43,46].

FISH is a rapid, accurate and also cost-effective method for the detection of *H. pylori* and determination of macrolide resistance in cultured *H. pylori* colonies. It can also be used directly on biopsy specimens for histopathological and microbiological examination^[12,47]. In this assay intact *H. pylori* are hybridized with fluorescent-labeled *H. pylori*-specific 16S and 23S rRNA probes. The labeled bacteria were subsequently visualized by fluorescence microscopy. This assay allows detection of *H. pylori* and clarithromycin resistance simultaneously. Moreover, this assay does not require DNA preparation and can directly be applied to gastric biopsy samples^[9,23,26,27,32,35,48].

For *H. pylori* a species-specific detection is performed by the 16S rRNA-specific oligonucleotide Hpy-1, labeled in green. Simultaneously to the species detection a genotypic antibiotic resistance determination is possible (labeled in red). The resistance against the macrolide clarithromycin, which is a major antibiotic used in the triple therapy against *H. pylori* infections, is based on three defined point mutations in the 23S rRNA. These point mutations can be targeted specifically with the ClaWT, ClaR1, ClaR2, and ClaR3 probes. Different mutations correlate with different MICs of the antibiotics, ClaR1 > 64 mg/L, ClaR2, and ClaR3 between 8 and 64 mg/L^[46,47].

CONCLUSION

The gold standard for accurate diagnosis of an *H. pylori* infection is either culturing of the pathogen or concordant positive results obtained by histology and the rapid urease test or the ¹³C-urea breath test (UBT)^[24]. After culturing for the pathogen of gastric biopsies, probably most laboratories use disk diffusion or E-test for the determination of macrolide resistance. Both methods require further sub-culturing for several days and cannot identify the type of point mutations present in the strain^[12].

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^[24,49]. Whilst the E-test is a phenotypic clarithromycin resistance measurement, FISH is an established genotypic technique for the detection of *H. pylori* and discrimination between the clarithromycin-susceptible wild type and clarithromycin-resistant mutants^[11,49]. FISH is a reliable fast method for the detection of clarithromycin-resistant *H. pylori* mutants, and results are available within 3 h after an endoscopy. The probes are commercially available, and the method is cost-effective and can be applied in any laboratory without the need for special equipment or facilities, except for a fluorescence microscope^[11,47,49].

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