**HELCOBACTER PYLORI**

**Routine use of real-time PCR for detection of Helicobacter pylori and of clarithromycin resistance mutations**


(1) Laboratoire de Bactériologie-Virologie, Hôpital Saint-Antoine and Centre Hospitalo-Universitaire Saint-Antoine, Assistance Publique-Hôpitaux de Paris, Université Paris VI, Paris ; (2) Département de Pathologie, Hôpital Henri-Mondor, Créteil ; (3) Service de Bactériologie-Virologie, Hôpital Henri-Mondor, Créteil ; (4) Service d’Hépatogastroentérologie, Hôpital Henri-Mondor, Créteil.

**SUMMARY**

Objectives — We previously showed that real-time PCR was a reliable technique for coupled detection of Helicobacter pylori and clarithromycin resistance mutations directly from biopsies. After one year of use, we compared its performances to those of histology, which remains the most employed method for H. pylori detection from gastric biopsies.

Materials and Methods — 518 subjects underwent endoscopy during the year 2003 with biopsies taken for H. pylori detection by gastric biopsies.

Results — The prevalence of infection, defined as positive PCR and histology, and in case of discrepancy as a positive culture, was 30% (163/518). The percentage of concordance between the two tests was 87.8% (455/518). The sensitivity, specificity, positive and negative predictive values of PCR were 98.2%, 97.5%, 94.7%, and 99.1%, respectively. The corresponding performances of histology were 87.7%, 91.3%, 82.2%, and 94.2%, respectively (p<0.001). The prevalence of clarithromycin resistance was 30%.

Conclusions — PCR is more accurate in routine than histology and permits easy determination of clarithromycin resistance, which is useful in countries like France where the prevalence of resistance is high.

**RÉSUMÉ**

**Utilisation en routine de la PCR en temps réel pour la détection de Helicobacter pylori et des mutations de résistance à la clarithromycine**

Jacques TANKOVIC, Marie-Thérèse CHAUMETTE-PLANCKAERT, Lionel DEFORGES, Nathalie LAUNAY, Jeanne-Marie LE GLAUNEC, Claude-James SOUSSY, Jean-Charles DELCHIER

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Objectif — Dans un travail préliminaire nous avions montré que la PCR en temps réel sur biopsies gastriques permettait de façon fiable de détecter à la fois Helicobacter pylori et d’éventuelles mutations de résistance à la clarithromycine. Après un an d’utilisation, nous avons comparé ses performances à celles de l’histologie, qui reste la technique de base pour la détection de H. pylori sur biopsies.

Matériel et Méthodes — 518 patients ont bénéficié en 2003 d’une endoscopie avec réalisation de biopsies pour la recherche de H. pylori par histologie, PCR, et en cas de discordance entre ces deux techniques, également par culture.

Résultats — La prévalence de l’infection, définie par la positivité de l’histologie et de la PCR ou, en cas de discordance, par la positivité également de la culture, était de 30 % (163/518). Le pourcentage de concordance entre les deux tests était de 87,8 % (455/518). Les sensibilité, spécificité, valeurs prédictives positive et négative étaient pour la PCR respectivement de 98,2 %, 97,5%, 94,7 % et 99,1 %. Les performances correspondantes de l’histologie étaient de 87,7 %, 91,3 %, 82,2 % et 94,2 % (p < 0,001). La prévalence de la résistance à la clarithromycine était de 30 %.

Conclusions — La PCR est plus fiable en routine que l’histologie et permet de tester facilement la sensibilité à la clarithromycine, ce qui est utile dans les pays à prévalence de résistance élevée, comme la France.

**Helicobacter pylori** is a gram-negative bacterium which chronically infects the stomach of approximately one half of the population worldwide and which is now recognized as the main cause of most gastroduodenal diseases [1, 2]. Numerous diagnostic assays for H. pylori detection are available, either invasive (requiring gastric endoscopy and biopsies) or non invasive ones [3, 4]. Invasive methods include culture, histology, rapid urease testing, and molecular assays.

Noninvasive methods include serology, urea breath testing, fecal antigen detection, and molecular assays on stool samples. Noninvasive methods are interesting but, with the exception of the last cited, do not provide information on resistance to antibiotics, and particularly to clarithromycin. Resistance to this drug is the main risk factor for failure of the standard triple therapy recommended in Europe, composed of this antibiotic plus amoxicillin and a proton pump inhibitor. It results in eradication rates of only 0 to 48% [5, 6]. One recommendation of the recent Maastricht III Consensus Report is that clarithromycin susceptibility testing should be performed before treatment when the prevalence of primary resistance reached 15-20% [7]. Such high prevalence rates already exist in several countries including France, Portugal, Italy, Mexico, the United States and Iran [8-10]. Furthermore,
clarithromycin resistance appears to be increasing at least in some countries [8].

Clarithromycin resistance is mostly due to one mutation in the 23S ribosomal RNA (rRNA) : adenine-to-guanine transition at positions 2142 or 2143 or adenine-to-cytosine transversion at position 2142. These two positions participate in the formation of the peptidyltransferase loop of the ribosome [9]. These mutations can easily be detected by molecular techniques, notably by real-time PCR, as we and others already showed [10-13]. Culture also permits to detect clarithromycin resistance but the disadvantages of culture are the need of special conditions for specimen transportation, the use of specific media and incubation conditions, and the delay necessary to obtain a result [14].

In a preliminary study, we found that real-time PCR with the LightCycler apparatus represented a fast, simple, and accurate alternative to culture for coupled detection of H. pylori and clarithromycin resistance directly from gastric biopsies [11]. This technique was thus introduced as routine in the laboratory of bacteriology of Henri-Mondor hospital in January 2003. This, to the best of our knowledge, constituted the first experience of routine use of PCR for the diagnosis of H. pylori and for clarithromycin resistance detection. Histology continued to be performed in routine, but not culture. This latter technique was only performed when sensitivity to other antibiotics than clarithromycin was required (before third-line treatment) or in case of discrepancy between PCR and histology.

Histology remains the most employed method for the diagnosis of H. pylori from gastric biopsies, but nowadays the bacterium is less frequently seen in biopsies even when chronic active gastritis is present [15]. One important explanation is certainly the frequent use of proton pump inhibitors and of antibiotics by patients in the weeks preceding the realization of endoscopy.

So the aims of this study were first to verify that, in routine use, real-time PCR was an accurate method for the detection of H. pylori from gastric biopsies and that it also permitted to test its susceptibility to clarithromycin. Of note, in this study the results of susceptibility testing by PCR were not compared with those obtained by microbiological methods, because this had already been demonstrated in the preliminary study. The second aim of that study was to confirm that real-time PCR was superior to histology for the detection of H. pylori.

Material and Methods

Study design

From January 1 to December 31, 2003, 518 subjects with a mean age of 54.1 years (range 17-88 years), 286 of whom were men (55.1%), underwent endoscopy with biopsy samples taken for the diagnosis of H. pylori infection and were thus included in the study. They represented roughly one third of all patients who underwent gastric endoscopy in Henri-Mondor hospital during the year 2003. The study patients had either nonulcer dyspepsia (322 patients, 62.2%), gastroduodenal ulcer or a history of it (123 patients, 23.7%), MALT lymphoma of the stomach (50 patients, 9.7%), other types of gastric lymphoma (17 patients, 3.3%), or gastric adenocarcinoma (6 patients, 1.1%).

Three endoscopic biopsies taken from the antrum (three cm from the pylorus) and three from the fundus were sent in formalin for histology. In addition, one biopsy from the antrum and one from the fundus were put into a sterile microtube, immediately frozen in liquid nitrogen, and stored at -80°C for PCR and eventually culture. Indeed, culture was performed only in case of discrepancy between histology and PCR, or when sensitivity to antibiotics other than clarithromycin was required (before third-line treatment).

Infection was defined as 1/positivity of PCR and histology or 2/positivity of one of the two methods plus positivity of culture.

Techniques used for histology, culture and PCR

Formalin-fixed mucosal samples were stained with cresyl fast violet at temperature of -80°C for PCR and eventually culture. Indeed, culture was performed only in case of discrepancy between histology and PCR, or when sensitivity to antibiotics other than clarithromycin was required (before third-line treatment).

PCR was performed twice a week with five samples per experiment in average as described previously [11]. Briefly, the antral and fundic biopsies were homogenized together in one ml of culture broth using single-use sterile plungers adapted for microtubes and half of the homogenate was used for PCR. Amplification was targeted at the 23S rRNA gene and the hybridization probe method was used for detection of the amplified product. Two separate PCR assays were performed: one for the detection of H. pylori and one for the detection of clarithromycin resistance mutations. DNA extraction was performed by using the High Pure PCR template preparation kit (Roche Molecular Biochemicals, Meylan, France). The absence of inhibitors of Taq DNA polymerase was verified by testing each DNA extract with or without addition of total DNA from H. pylori.

For culture, the congelated other half of the homogenate was used. After decongelation, it was plated onto on a commercialized serum-supplemented selective medium, Pylori agar (bioMérieux, Marcy l’Etoile, France), under microaerophilic conditions at 37°C for a maximum of seven days. H. pylori isolates were defined as gram-negative spiral-shaped bacilli that were oxidase positive and rapidly (less than one hour) urease positive.

Statistical analysis

The Pearson’s Khi-2 test and the Mac Nemar’s test were used to compare the performances of PCR and histology and to analyze the discrepancies between the two techniques, respectively. Differences resulting in p-values of less than 0.05 were considered significant.

Results

Prevalence of infection

The prevalence of infection was 31.5% (163/518). As expected, the highest prevalence was observed for patients with active duodenal ulcer (table I). The prevalence was very low for patients with MALT lymphoma (table I). This is explained by the fact that our centre is specialized in the management of this illness and so the great majority of the patients with MALT lymphoma included in the study had already benefited from a successful eradication of H. pylori.

Compared performances of real-time PCR and histology

As shown in table II, the performances of PCR (sensitivity, specificity, positive and negative predictive values) were significantly better than those of histology (p<0.001). The percentage

<table>
<thead>
<tr>
<th>Disease</th>
<th>Prevalence of infection</th>
</tr>
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<tbody>
<tr>
<td>Duodenal ulcer</td>
<td>71% (34/48)</td>
</tr>
<tr>
<td>Gastric ulcer</td>
<td>46% (11/24)</td>
</tr>
<tr>
<td>Healed gastroduodenal ulcer or past history of gastroduodenal ulcer</td>
<td>25% (13/51)</td>
</tr>
<tr>
<td>Non ulcer dyspepsia</td>
<td>31% (99/322)</td>
</tr>
<tr>
<td>MALT lymphoma</td>
<td>2% (1/50)</td>
</tr>
<tr>
<td>Other types of gastric lymphoma</td>
<td>24% (4/17)</td>
</tr>
<tr>
<td>Gastric carcinoma</td>
<td>17% (1/6)</td>
</tr>
</tbody>
</table>
Colonies were present on the plates.

Denal ulcer disease is increasing [16-18]. But we did not verify if the patients had taken antibiotics or antisecretory drugs in the weeks preceding endoscopy.

The well-know fact that the proportion of infection missed by histology (table III), only five (25%) were found H. pylori-positive by the second reading. Concerning the cases classified as false positivities on histology, almost all were confirmed negative by the second reading (29/31, 96%).

There were nine cases of isolated positivity of PCR. A blinded second reading of histology was also performed for these cases and two were corrected as histology-positive. There were also three cases of infection that were missed by PCR (table II) and we verified that this was not due to the presence of Taq polymerase inhibitors. In two cases, the culture was very weak: one and two colonies were present on the plates.

**Clarithromycin susceptibility testing by real-time PCR**

Clarithromycin susceptibility testing by PCR could be done in almost all cases (97%, 158/163). In five cases, the melting curves were not readable. The prevalence of resistance in Henri-Mondor hospital for the year 2003 was 27.2% (43/158). However, this value does not reflect primary resistance to clarithromycin because a notable proportion of the patients included in the study had already received one or more courses of eradication therapy.

**Feasibility and cost of PCR**

Two assays were performed per week with five samples per assay in average. The duration of PCR was two hours, including the extraction step, with one additional hour in case of positivity for the realization of the second PCR dedicated to the detection of clarithromycin resistance mutations. The cost per patient was evaluated at 12.3 € for the two PCRs (detection of the bacterium and detection of the mutations) whereas that of culture and clarithromycin susceptibility testing by E-test was 6.0 €.

**Discussion**

In this study we showed that real-time PCR was routinely capable to accurately detect H. pylori directly from gastric biopsies and also to determine the susceptibility of the strain to clarithromycin. The prevalence of infection was 31.5%; it was 71% for patients with active duodenal ulcer. This is in agreement with the well-know fact that the proportion of H. pylori-negative duodenal ulcer disease is increasing [16-18]. But we did not verify if the patients had taken antibiotics or antisecretory drugs in the weeks preceding endoscopy.

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**Table II.** Performances of real-time PCR and histology.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Real-time PCR</th>
<th>Histology</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>98.2% (160/163)</td>
<td>87.7% (143/163)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Specificity</td>
<td>97.5% (346/355)</td>
<td>91.3% (324/355)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Positive Predictive Value</td>
<td>94.7% (160/169)</td>
<td>82.2% (143/174)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Negative Predictive Value</td>
<td>99.1% (346/349)</td>
<td>94.2% (324/344)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

*a Infection was defined as *POSITIVITY* of the two techniques, and in case of discrepancy as *POSITIVITY* also of culture.

**Table III.** Comparison of the results of real-time PCR, histology, and culture for the diagnosis of H. pylori infection.

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Real-time PCR</th>
<th>Histology</th>
<th>Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>140</td>
<td>+</td>
<td>-</td>
<td>- done</td>
</tr>
<tr>
<td>315</td>
<td>-</td>
<td>-</td>
<td>- done</td>
</tr>
<tr>
<td>20</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>31</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

We found that the performances of real-time PCR (sensitivity, specificity, positive and negative predictive values) were significantly better than those of histology. There were 63 discrepant results (12% of all the cases) and there was a trend towards better accuracy of PCR for these discrepant results but which did not reach statistical significance. One limitation of our study is that discrepancies between PCR and histology were classified as “positive” or “negative” according to the result of only one method: culture. Furthermore, culture was performed secondarily, after decongelation of the homogenate, which probably reduced at least slightly the sensitivity of culture. Our method of evaluation would have been more precise if more diagnostic tests had been performed, like serology or urea breath test.

The results of the blinded second reading performed by a specialized histologist confirmed the lower sensitivity of histology compared to PCR and showed that in most cases it was not observer-dependent. In agreement with that, the sensitivities of histology in that study and in the preliminary one [11] were identical (88%), although in the previous study all the primary readings were made by the same specialized pathologist. This blinded second reading also showed that histology can suffer from a lack of specificity which appears to be notably observer-dependent.

There were nine cases of isolated positivity of PCR, which were decreased to seven after the blinded second histology.

Three of these seven cases were strongly positive by PCR and thus probably corresponded to true positives whereas four cases were weakly positive (positivity detected after 39 cycles of amplification) and could correspond either to false negatives or true positives. There were also three cases of false negativity of PCR. These negative results could be explained by a poor quality of DNA extraction, but it was not examined further. So, we were not able to clearly characterize most cases of isolated positivity or negativity of PCR. More diagnostic tests would have helped to evaluate these discrepant results.
In some non routine studies, the superiority of conventional PCR over histology has already been reported [19-21]. In another study, conventional PCR was found to be a very sensitive method, much more than histology, for detecting the bacterium in bleeding peptic ulcers [22]. This aspect was not evaluated in our study. Conventional PCR was found to be less sensitive than histology in only one study but histology was performed using Warthin-Starry stains, which are time-consuming and difficult to prepare, thus not suitable for routine use [23]. This work, to our knowledge, is the first which compared the performances in routine of histology and real-time PCR for the detection of *H. pylori*.

Concerning the detection of clarithromycin resistance, our work demonstrated its feasibility by real-time PCR. In that study, we did not evaluate its correlation with susceptibility testing by a microbiolocal method, but this was done during the preliminary study [11] and with excellent results, 98.2% concordance (55/56). Other workers also demonstrated the reliability of this molecular technique for the detection of clarithromycin resistance [12, 13].

The high prevalence of resistance found in our study (27.2%) justifies our decision of systematic clarithromycin susceptibility testing, as recommended by the Maastricht III Consensus Report [7]. Furthermore, the prevalence found in this study is higher than that found in the preliminary study [11] performed in our hospital with strains isolated in 2000-2001 (originating from patients untreated or already treated, similarly to the present study) : 18.5% (12/65). A slightly increased prevalence in Henri-Mondor hospital has also been observed in 2004-2005 compared to 2003: 28.5% (35/123) [24]. This increasing resistance is not specific to our hospital, according to the prevalence of primary resistance to clarithromycin reported in a French multicenter study performed with strains from 1996-1997 and 1999-2000 : 14.3% and 18.4%, respectively [25]. Furthermore, this phenomenon is not restricted to France. Prevalences of resistance to clarithromycin similar to that experienced in France and increase in the prevalence of resistance have also been observed in several European and non-European countries [8-10]. Thus, clarithromycin susceptibility testing should be performed before treatment in many countries. The PCR technique we use is well suited for that purpose if endoscopy is performed.

The feasibility of this real-time PCR assay was good, with a duration of two to three hours and its cost was reasonable, twice the price of culture plus clarithromycin susceptibility testing by Etest. However, clarithromycin susceptibility testing is also possible with an erythromycin disk, at a lesser cost. Concerning the price of the thermocycler, this technique does of course not require a specific apparatus. It can be performed on any Light Cycler apparatus available in the laboratory. The technique can also be adapted to other types of real-time thermocyclers.

In conclusion, our work shows that the real-time PCR technique that we have introduced in routine in our hospital is easy to perform and more accurate than histology for the detection of *H. pylori*. Furthermore, it permits a concomitant easy and reliable determination of clarithromycin susceptibility, which we think is now mandatory in countries like France where the prevalence of resistance to clarithromycin is high, at least 15%, and increasing.

**REFERENCES**