Usefulness of Housekeeping Genes for the Diagnosis of Helicobacter pylori Infection, Strain Discrimination and Detection of Multiple Infection

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Abstract

Background: Helicobacter pylori infects human stomachs of over half the world’s population, evades the immune response and establishes a chronic infection. Although most people remains asymptomatic, duodenal and gastric ulcers, MALT lymphoma and progression to gastric cancer could be developed. Several virulence factors such as flagella, lipopolysaccharide, adhesins and especially the vacuolating cytotoxin VacA and the oncoprotein CagA have been described for H. pylori. Despite the extensive published data on H. pylori, more research is needed to determine new virulence markers, the exact mode of transmission or the role of multiple infection.

Materials and Methods: Amplification and sequencing of six housekeeping genes (amiA, cgt, cpn60, cpn70, dnaJ, and luxS) related to H. pylori pathogenesis have been performed in order to evaluate their usefulness for the specific detection of H. pylori, the genetic discrimination at strain level and the detection of multiple infection. A total of 52 H. pylori clones, isolated from 14 gastric biopsies from 11 patients, were analyzed for this purpose.

Results: All genes were specifically amplified for H. pylori and all clones isolated from different patients were discriminated, with gene distances ranged from 0.9 to 7.8%. Although most clones isolated from the same patient showed identical gene sequences, an event of multiple infection was detected in all the genes and microevolution events were showed for amiA and cpn60 genes.

Conclusions: These results suggested that housekeeping genes could be useful for H. pylori detection and to elucidate the mode of transmission and the relevance of the multiple infection.

Helicobacter pylori chronically infects more than half of the world’s population because the host immune response fails to eliminate the infection. In most infected people, the bacterium acts as a commensal organism inducing chronic asymptomatic gastritis that can last for life. In other cases, however, it is responsible for a heavy toll of morbidity and mortality as a consequence of peptic ulcers and gastric cancer. Chronic gastritis may progress to intestinal metaplasia, dysplasia and eventually gastric cancer. This multi-step process is known as the Correa pathway [1]. The clinical outcome of H. pylori infection depends on strain virulence, host response, and environmental factors.

Several virulence factors have been identified in H. pylori: gastric colonization mediated by flagella, urease, outer membrane proteins (OMPs), phospholipids, glycolipids, and other adhesins (babA, hpA, napA, sabA, etc.), toxins such as lipopolysaccharide (LPS) and the vacuolating cytotoxin VacA, a type IV secretion system encoded by the cag pathogenicity island (cagPAI) containing the effector protein CagA, a γ-Glutamyl transpeptidase (GGT) associated with colonization and
cell apoptosis, the duodenal ulcer-promoting gene A (dupA), and others [2–4]. Among virulence factors identified in H. pylori, cagA, and vacA genes are the main virulence markers as shown in an study conducted by [5], in which patients infected with high virulence strains (cagA+, vacA s111m1) had a higher risk of progression to preneoplastic lesions and gastric cancer in comparison with patients infected with low virulence strains.

Strains of H. pylori exhibit considerable genetic diversity following a panmictic (non-cloneal) population structure due to horizontal gene transfer and frequent recombination. In contrast, since H. pylori does not spread epidemically, phylogenetic studies based on multilocus sequence analysis (MLSA) of housekeeping genes are contributing to trace human migrations [6,7]. On the other hand, intrapatient diversity of H. pylori needs to be reevaluated because results about the prevalence of multiple infection are controversial, and it could have implications related to the mode of transmission, antibiotic resistance and virulence of H. pylori [2,8,9]. Moreover, there is a lack of studies regarding H. pylori detection from specimens other than gastric biopsies [10].

Other genes have likewise been related to H. pylori pathogenesis: amiA (N-acetylmuramoyl-L-alanine amidase) and luxS (autoinducer-2 synthase) genes are involved in biofilm formation although the former is also related to bacterial adhesion, cell morphology and immune escape [11]. Others such the cholesterol-α-glucosyltransferase gene (cgt or capJ) glycosilates host cholesterol contributing to pathogenicity and antimicrobial resistance while cpn40 (dnaJ), cpn60 (groEL), and cpn70 (dnaK) genes have been suggested as a risk factor for oncogenesis and are recommended as serological markers of H. pylori infection [3,12,13].

In this study, H. pylori specific PCR amplification and sequencing of amiA, cgt, cpn60, cpn70, dnaJ, and luxS genes have been developed in order to evaluate their usefulness in the detection of H. pylori infection, to genetic discrimination at strain level, to detection of multiple infection and to MLSA.

Materials and Methods

Helicobacter pylori Clones and DNA Extraction

Ten H. pylori strains (APP134, B247, B271, B319, B355, B491, B508S, B508T, CRL122, and SVC135) from the H. pylori collection of the Digestive Diseases Department of the Hospital Taulí (Sabadell, Barcelona, Spain) were included in this study. Outpatients sent to the Endoscopy Unit of the Hospital Taulí for evaluation of dyspeptic symptoms from February 2006 to November 2015 were recruited in this collection. Patients were contacted prior to the endoscopy and were asked to participate. Before the endoscopy, the patients signed informed consent. During endoscopy, antral and corpus biopsies were obtained for histology, rapid urease test (RUT) and molecular studies. Isolation, culture and identification of H. pylori were performed after a positive RUT test. The RUT biopsy was plated on Pylori Agar (bioMérieux, Marcy l’Étoile, France) in microaerophilic conditions in microaerophilic jars (Jar Gassing System; Don Whitley Scientific Limited, Shipley, West Yorkshire, UK). After a maximum of a week, H. pylori isolates were subcultured on Columbia plates (bioMérieux) and identified by colony morphology, Gram-negative staining and a positive result for urease, catalase, and oxidase tests. The strains were frozen in Brucella Broth with 10% glycerol and stored at −80 °C until analysis [14].

The strains were recovered on Columbia agar with 5% sheep blood (bioMérieux) and incubated at 37 °C under microaerophilic conditions. These strains were previously isolated from antral biopsies of different patients, except for B508S and B508T, which were isolated from the same patient, B508S from normal tissue and B508T from gastric adenocarcinoma (Table 1). For this study, these H. pylori strains were not considered as pure cultures because they were originally obtained by picking up most of the growth on Pylori agar (bioMérieux) after seeding of gastric biopsies, and not

| Gastric biopsies (n = 14) | Endoscopic/ Histopathological diagnosis | Clones analyzed (n = 52) |
|---------------------------|---------------------------------------|-------------------------|
| APP134                    | Duodenal ulcer/mild gastritis          | APP134-1, -2, -3        |
| B247                      | Neoplasia/adenocarcinoma               | B247-1, -2, -3          |
| B271                      | Duodenal ulcer/mild gastritis          | B271-1, -2, -3          |
| B319                      | Normal/mild gastritis                  | B319-1, -2, -3          |
| B355                      | Duodenal ulcer/mild gastritis          | B355-1, -2, -3          |
| B491                      | Neoplasia/adenocarcinoma               | B491-1, -2, -3          |
| B508S                     | Neoplasia/adenocarcinoma               | B508S-1, -2, -3, -4, -5, -6 |
| B508T                     | Neoplasia/adenocarcinoma               | B508T-2A, -2B, -3, -4, -5, -6 |
| B657A                     | Normal/mild gastritis                  | B657A-1, -2, -3, -4    |
| B657C                     | Normal/mild gastritis                  | B657C-1, -2, -3, -4    |
| B659A                     | Normal/moderate gastritis              | B659A-1, -2, -3, -4    |
| B659C                     | Normal/moderate gastritis              | B659C-1, -2, -3, -4    |
| CRL122                    | Normal/mild gastritis                  | CRL122-1, -2, -3       |
| SVC135                    | Normal/moderate gastritis              | SVC135-1, -2, -3       |

*Gastric biopsies obtained from normal tissue of patients with adenocarcinoma.

Table 1 Samples included in this study
from individual colonies. In order to obtain pure cultures, between three and six isolated colonies were selected from each blood agar culture.

*Helicobacter pylori* colonies were also isolated from antral and corpus biopsies of two different patients (B657A/B657C and B659A/B659C) (Table 1) seeded onto Pylori agar.

*Helicobacter pylori* strains ATCC 49503 and ATCC 51932 were also included as controls in the present study.

Subcultures of individual colonies were performed on Columbia blood agar or Brucella agar (BD Diagnostics, Franklin Lakes, NJ, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Waltham, MA, USA).

**DNA Extraction, PCR Amplification and Sequencing**

DNA of each clone was extracted from 4 ml of cultures in Brucella broth supplemented with 10% FBS following the manufacturers’ instructions (REAL pure genomic DNA extraction kit; Durviz, Paterna, València, Spain).

Primers for *H. pylori* specific PCR amplification and sequencing of the housekeeping genes *amiA*, *cgt*, *cpn60*, *cpn70*, *dnaJ*, and *luxS* (Table 2) were designed from 43 *H. pylori* complete genome sequences (see Table S1). To evaluate the specificity of the PCR assays, the following four strains, belonging to different bacterial species, were included as negative controls: *Campylobacter jejuni* ATCC 33291, *Pseudomonas aeruginosa* 42A2, *Aeromonas molluscorum* 848TT, and *Aeribacillus pallidus* DR03.

PCR amplifications were carried out in a total volume of 50 µL containing 50 mM KCl, 15 mM Tris/HCl, pH 8.0, 2.5 mM MgCl$_2$, 0.2 mM dNTPs (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Waltham, MA, USA), 40 pmol of each primer (Sigma, St. Louis, MO, USA), and 250 ng DNA. The reaction mixtures were subjected to the following thermal cycling program in a 2720 Thermal Cycler (Applied Biosystems): denaturation at 95 ºC for 5 minutes, 35 cycles of 95 ºC for 1 minutes, 54–64 ºC (Table 2) for 1 minute and 72 ºC for 1 minute and a final extension step at 72 ºC for 5 minutes.

The amplified products were purified using the MSB® Spin PCRapace kit (Strattec, Birkenfeld, Germany) or the ExoSAP-IT® (Affymetrix, Santa Clara, CA, USA) and sequencing was performed using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) with the following thermal cycling conditions: 96 ºC for 1 minute and 25 cycles of 96 ºC for 10 seconds, 50 ºC for 5 seconds and 60 ºC for 4 minutes. Nucleotide sequences were determined in an ABI PRISM 3730 DNA analyzer by the Genomics Unit of Scientific and Technological Centers from University of Barcelona (CCiTUB).

**Data Analyses**

Partial sequences of *amiA*, *cgt*, *cpn60*, *cpn70*, *dnaJ*, and *luxS* genes were aligned independently and phylogenetic trees were constructed by neighbor-joining method and Jukes–Cantor distance estimation model using MEGA 6.0 [15]. Concatenated sequences of all genes obtained by the online sequence toolbox FaBox [16] were also analyzed (Table 3). The topological robustness of the phylogenetic trees was evaluated by a bootstrap analysis through 1000 replicates.

| Gene | Length (pb)* | Primer | Sequence 5’→3’ | Ta | Amplicon (pb) |
|------|-------------|--------|----------------|----|---------------|
| amiA | 1323        | amiA-658 | GTTTTTGAGACCGYGGGAGATGC | 64 ºC | 635 |
|      |             | amiA-1292 | TTTATGAAACCGCTTTTTTGTG | 60 ºC | 615 |
| cgt  | 1170        | cgt-252  | GGTCTTGAAGGAGCCTTGGATGA | 60 ºC | 615 |
|      |             | cgt-866  | ATCGCTTGCCTTGTCCCACATT | 54 ºC | 801 |
| cpn60| 1641        | Hp156   | CGTAGGCCTTGGCTTAARGAG | 54 ºC | 801 |
|      |             | Hp956   | GCTTGGCTAAAGACTCYACTT | 54 ºC | 801 |
| cpn70| 1863        | cpn70-982 | ATTTCAAGATGTGGTATGT | 54 ºC | 612 |
|      |             | cpn70-1593 | GTTTCTCGCTTCAATCACTT | 54 ºC | 588 |
| dnaJ | 1110        | dnaJ-259 | TTAGGATTTAGGCTTCCTT | 54 ºC | 429 |
|      |             | dnaJ-846 | TAAAGACCGGACCTTAAATGG | 54 ºC | 429 |
| luxS | 468         | luxS-38  | TGGTACATCAACAAAGTTGCAAAG | 54 ºC | 429 |
|      |             | luxS-466 | TAACCCCACCTTCAGACCA | 54 ºC | 429 |

| Ta, annealing temperature. |
| *Data from the complete genome of *Helicobacter pylori* 26695 (NC_000915).
sequence comparison, all B508T and B508S clones ana-
tance values were obtained from.

distances ranged from 0.9 to 7.8% and the highest dis-
biopsies of different patients were different, whose gene 
pared. All gene sequences from clones isolated from 
and B657A/B657C. Two different 
identical sequences except in the case of B508S/B508T 
2.4 to 3.6%, with a mean distance of 3% (Table 3).
calculated from concatenated sequences ranged from 
H. pylori 
amiA 

Table 3 Genetic distance values of individual and concatenated genes

| Gene   | Sequence (nt) | Distances values |  
|--------|---------------|------------------|
|        |               | Range      | Minimuma | Mean ± SE  |
| amiA   | 576           | 0–0.052  | 0.019    | 0.031 ± 0.004 |
| cgt    | 558           | 0–0.044  | 0.009    | 0.025 ± 0.004 |
| cpn60  | 555           | 0–0.039  | 0.009    | 0.023 ± 0.004 |
| cpn70  | 588           | 0–0.040  | 0.015    | 0.027 ± 0.004 |
| dnaJ   | 564           | 0–0.061  | 0.013    | 0.032 ± 0.004 |
| luxS   | 405           | 0–0.078  | 0.028    | 0.046 ± 0.006 |
| Concatenated | 3246  | 0–0.036  | 0.024    | 0.030 ± 0.002 |

aMinimum distances values between sequences from clones isolated from biopsies of different patients.

Results

A total of 52 H. pylori clones were isolated from 14 gas-
tric biopsies from 11 patients (Table 1).

All housekeeping genes (amiA, cgt, cpn60, cpn70, 
dnaJ, and luxS) were specifically amplified for the bacte-
rial species H. pylori with the primers designed in this 
study, none gene was amplified for the other bacterial 
species analyzed. Amplicons and partial gene sequences 
were found in 

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Discussion

In this study, specific PCR detection of H. pylori has been 
achieved for the housekeeping genes amiA, cgt, 
cpn60, cpn70, dnaJ, and luxS. These genes are potential 
candidates to detect H. pylori infection in gastric biop-
sies and other specimens (as gastric juice, stool, saliva, 
dental plaque, water, and food samples), together with 
others, mainly used for gastric biopsies, as ureA, vacA, 
16S rRNA, and 23S rRNA genes, previously described 
[17,18].

Sequencing of any of these six genes has allowed a 
clear differentiation between all clones isolated from dif-
ferent patients (0.9–7.8% divergence). These results sug-
gest that these housekeeping genes could be useful to 
elucidate the mode of transmission of H. pylori, an issue 
that is still controversial. While oral-oral, gastro-oral, and 
fecal-oral transmission are the most probable ways, H. pylori 
isolation from stools or the oral cavity is difficult and 
different environmental sources of H. pylori (food, 
animals, water) could play a role as reservoirs [10].

Two different strains isolated from the antral biopsy 
B657A were clearly detected by all genes with high dis-
ance values (1.6–7.3%). One strain was represented by 
the identical clones B657A-1, -2 and -3, and the other 
strain by the clone B657A-4, which was identical to 
clones isolated from B657C (corpus biopsy from the 
same patient), except in the case of amiA as mentioned 
below. This high divergence observed between both 
strains indicates an event of multiple infection in the 
antrum of the stomach. Horizontal gene transfer and 
genetic recombination by mixed infection is crucial for 
the acquisition of the high genetic diversity of H. pylori

Figure 1 Consensus neighbor-joining phylogenetic trees obtained from 18 Helicobacter pylori sequences of genes amiA (A) cpn60 (B) and luxS (C). Bar, distance values as calculated by MEGA 6.0. Bootstrap values (>50%) after 1000 replicates are shown as percentages.
Although some authors have detected mixed infections, different studies have reported very variable prevalence rates and little is known about the role of multiple infections on disease outcome [8,20,21]. Although luxS gene showed the highest distance values, the genes amiA and cpn60 were more useful to discriminate clones isolated from the same biopsy and patient. Two different clones isolated from the sample B508T, gastric biopsy collected from tissue with adenocarcinoma, were only detected by partial sequencing of amiA and cpn60 (one and six nucleotide differences, respectively). One of both clones (B508T-4) was identical to clones isolated from B508S, gastric biopsy from the same patient but collected from normal tissue. On the other hand, two amiA nucleotide differences were observed between B657A-4 and B657C clones. These small differences between clones suggest events of microevolution rather than related to multiple infection. Evidences that H. pylori can establish a chronic infection after infection with a single strain have been described [22,23] and, recently, Linz et al. [24] have revealed that the mutation rate during the acute infection phase is over 10 times faster than during chronic infection.

Concatenation of amiA, cgt, cpn60, cpn70, dnaJ, and luxS partial sequences allowed us to conduct a multilocus sequence analyses (MLSA) approach (Fig. 2), which is useful to elucidate intra- and interspecies phylogenetic relationships [25,26] and, in the case of H. pylori, the phylogeographic differentiation of bacterial populations associated to the migration of human populations [6,27]. Most of these genes have never been used for these purposes except dnaJ and, especially, cpn60, that is useful for microbial phylogeny, detection and identification, ecology, and evolution through the analysis of the 555 bp region known as universal target (UT) analyzed in this study [28,29].

In conclusion, H. pylori specific detection has been developed, all H. pylori clones isolated from different patients have been discriminated, and microevolution and mixed infection have been detected by means of amplification and sequencing of housekeeping genes amiA, cgt, cpn60, cpn70, dnaJ, and luxS for the first time. Further studies based on these housekeeping genes are needed to explore their potential applications for H. pylori detection in different specimens, the mode of transmission of H. pylori, the role associated to virulence or the phylogeographic differentiation of H. pylori populations.

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Competing interests: The authors have declared that no competing interests exist.

Note

The GenBank/EMBL/DDBJ accession numbers for the amiA, cgt, cpn60, cpn70, dnaJ, and luxS gene sequences are KU053341–58, KU053359–76, KU053377–94, KU053395–412, KU053413–30 and KU053431–48, respectively.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Consensus neighbor-joining phylogenetic trees obtained from 18 H. pylori sequences of genes cgt (A), cpn70 (B), and dnaJ (C). Bar distance values as calculated by MEGA 6.0. Bootstrap values (>50%) after 1000 replicates are shown as percentages.

Table S1 Helicobacter pylori complete genome sequences used to primer design.

Palau et al.

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