Detection of virulence gene belonging to cag pathogenicity island in Helicobacter pylori isolates after multiple unsuccessful eradication therapy in Northwest Croatia

Abstract

Background: Some of the genes belonging to cag pathogenicity island (cagPAI) in Helicobacter pylori were found to be associated with an increased severity of gastric mucosal inflammation that might lead to the development of gastroduodenal disease.

Aim: The aim of our study was to define a group of patients based on the frequency of virulence genes of cagPAI island and comparison with pathological alterations of gastric mucosa who need to be subjected to further eradication therapy after previous unsuccessful eradication therapy and in spite of benign endoscopic findings.

Material and methods: In total 103 H. pylori isolates were analysed. Genes encoding virulence factors were detected by PCR with primers for 10 loci in cagPAI: Ap\(cag\) (cag\(A\) promotor region), cag\(A1\), cag\(A2\), cag\(A3\), cag\(M\), cag\(E\), LEC, tnp\(A\) and tnp\(B\). The patients who provided isolates were classified into three clinical categories: non-ulcer dyspepsia (n=69), erosio/ulcus ventriculi (n=22) and erosio/ulcus duodeni (n=12).

Results: 16 strains (15.5%) were negative for all tested genes. 87 (84.5%) of the isolates had partially deleted cagPAI. None of the isolates possessed all 10 genes. The frequency of single cagPAI genes were as follows: Ap\(cag\) 63.1%, cag\(A1\) 71.8%, cag\(A2\) 69.9%, cag\(A3\) 5.8%, cag\(M\) 71.8%, cag\(E\) 75.7%, cag\(T\) 68%, tnp\(A\) 9.7%, tnp\(B\) 7.8% i LEC 48.5%.

No statistically significant difference was observed between the presence of any cagPAI genes and endoscopic diagnosis (p>0.16). The presence of CagA2, Ap\(cag\) and cagM showed statistically significant correlation with higher level of pathohistological parameters of gastritis (p<0.05).

Conclusions: H. pylori isolates with positive cag\(A\), Ap\(cag\) and cag\(M\) genes are correlated to higher degree of pathohistological lesions of gastric mucosa; without statistically significant correlation with endoscopic diagnosis.

INTRODUCTION

Many studies have confirmed the role of H. pylori in the development of chronic gastritis, gastric and duodenal ulcer and the ethiological role in the pathogenesis of gastric carcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma.

H. pylori possess many virulence genes; one of them is cag pathogenicity island (cagPAI) (1). CagPAI is since its discovery the most analysed
segment of \textit{H. pylori} genome. It is approximately 40 kilo-
base pairs region of \textit{H. pylori} chromosome and contains
around 30 genes divided into region I and II. \textit{CagPAI} is
defined as intact if all genes are present, partially deleted
as presence of several genes and negative (deleted) if there
are no genes at all (2). Many studies report correlation
between presence of intact \textit{cagPAI}, partially deleted and
deleted \textit{cagPAI} and clinical outcome. Some authors cor-
relate intact \textit{cagPAI} with severe gastroduodenal diseases,
with higher grade of chronic gastritis and premalignant
lesions of gastric mucosa. \((1, 3–4)\), while partially de-
leted and deleted \textit{cagPAI} are associated with milder
forms of gastroduodenal disease and lower grade of path-
ological alternations of gastric mucosa \((5–6)\). On the
contrary, some of the authors did not find correlation be-
tween \textit{cagPAI} and gastroduodenal disease \((7)\). \textit{CagPAI}
encodes multiple structural components of bacterial type
IV secretion system (T4SS). T4SS translocates \textit{cagA} pro-
tein directly to the cytosol of the gastric epithelium where
it gets tyrosin phosphorylated by Src-family kinases and
becomes able to alter the host cell functions leading to
malignant transformation \((2)\).

\textit{CagA} gene is located in the region I of \textit{cagPAI} and is
considered to be the marker of this region. \textit{CagA} positive
isolates are associated with more severe clinical features
in many studies. However, there are contradictory results
in the references regarding these studies.

\textit{CagE} is also located in the region I and is necessary to
induce production of interleukin IL-8. Some authors con-
sider \textit{cagE} gene to be better marker of \textit{cagPAI} region com-
pared to \textit{cagA} and more useful in monitoring the progress
of \textit{H. pylori} induced gastric disease. \textit{CagT} gene is a mark-
er of \textit{cagII} region and some studies connect it with more
severe clinical disease \((8)\). LEC (left terminal end of \textit{cagII})
is not necessary for translocation of \textit{cagA} into the host cell
or induction of interleukin IL8. It is associated with pep-
tic ulcer and adenocarcinoma. Some of the study found
connection of \textit{tnpA} gene with peptic ulcer \((9)\).

There are no published studies on the presence of \textit{H.
pylori} virulence genes in Croatia. Considering quite large
number of patients with multiple unsuccessful eradication
therapy, in spite of lack of clinical symptoms and benign
endoscopic result, there is a question to pose whether to
insist on eradication or not.

The aim of our study was to detect virulence genes of
\textit{H. pylori} just in these patients as a possible predictors of
future severe gastroduodenal diseases, by comparing it
with clinical and pathohistological results.

\section*{MATERIAL AND METHODS}

\subsection*{Patients}

The study analysed gastroscopic test results and bioptic
specimens of gastric mucosa with positive \textit{H. pylori} culture
in 103 patients examined during routine, clinical gastro-
duodenoscopies in the endoscopic laboratory of the Uni-
versity Hospital Merkur in Zagreb during the period
2008–2012. Microbiological and molecular analysis was
performed at the Department for Clinical and Molecular
Microbiology of the University Hospital Center in Zagreb
and pathohistological testing of the gastric biopsy speci-
mens at the Department for Pathology of University Hos-
pital Merkur. The study was approved by the Ethical Co-
mmittee of the University Hospital Center Zagreb and
University Hospital Merkur. The patients had signed the
informed consent. Twenty-six men and seventy-seven
woman in the age range of 28 to 80 years were included
in the study. All patients were previously treated with
eradication therapy for \textit{H. pylori}. According to the endo-
scopic finding patients were classified in three groups: non
ulcer dyspepsia (NUD), erosio/ulcus ventriculi (EUV),
erosio/ulcus duodeni (EUD).

\subsection*{Bacterial culture}

The biopsy specimens (one from corpus and one from
antrum) were transported in tioglicolate broth, homog-
ized and seeded on Columbia agar with addition of 7%
horse blood and \textit{Helicobacter pylori} Selective Supplement
SR 0147E (Oxoid) for cultivation of \textit{H. pylori}. The plates
were incubated in microaerophylic atmosphere with
100% humidity for 3–5 days. Identification was done
based on macromorphology, micromorphology and bio-
chemical testing (oxidase, urease, catalase). The strains
were stored at \(-80\)°C in brucela broth with 10% glycerol.

\subsection*{DNA extraction}

Extraction of chromosomal DNA was performed with
commercial kit: High Pure PCR Template Preparation
Kit, Version 16 (Roche Diagnostics GmbH, Mannheim,
Germany) according to the manufacturer’s recommenda-
tion. The DNA was stored at \(-20\)°C until used for mo-
lecular studies.

\subsection*{PCR amplification}

PCR was used to detect the following genes: \textit{cagA1},
\textit{cagA2}, \textit{cagA3}, \textit{cagE}, \textit{cagM}, \textit{cagT}, \textit{cagA} promotor region (Ap-
cag), \textit{tnpA}, \textit{tnpB} and LEC using primers and conditions
shown in Table 1. \((10–14)\). A set of primers P1 and P2 that
amplified a 26 kDa antigen \((Ag)\) gene present in all strains
of \textit{H. pylori} was used as a positive PCR control. All PCR
reactions were performed using a GeneAmpC PCR System
9700 (A6B Applied Biosystems). PCR products were visu-
alised by electrophoresis in in 2% agarose gel, after staining
with ethidium bromide and examined in UV transillumi-
nator. A 100 bp DNA ladder (Sigma) was used as a size
marker. Reference strains 47164 and 17874 (Culture col-
mercial kit: High Pure PCR Template Preparation
Kit, Version 16 (Roche Diagnostics GmbH, Mannheim,
Germany) according to the manufacturer’s recommenda-
tion. The DNA was stored at \(-20\)°C until used for mo-
lecular studies.

\subsection*{Histology}

Specimens for pathohistological analysis were fixed in
a standard 4% neutral buffered formalin, and cut into
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Slides. Slides were routinely stained and analysed according to modified Sydney classification of gastritis (15). Metaplasiae were scored as yes or no, concerning that none of the patients had incomplete metaplasia of II or III grade. All metaplasias were of the I grade.

**Statistical analysis**

Age and complex scores were expressed as mean and standard deviation (SD). Comparison for complex scores were done using Student’s t-test. Categorical variables were presented as frequencies (%). The comparison between subgroups for categorical variables were done using χ² test or Fischer exact test with the calculation of odds ratio (OR) together with 95% confidence intervals (CI). Logistic regression analysis was used to calculate OR (95%CI) for the association of the presence of individual genes with complex scores (OR was calculated for the 1-point change in complex score). A P value of <0.05 was considered statistically significant for all tests performed. The analysis was performed using STATISTICA, version 10. (StatSoft, Inc., OK, USA).

**RESULTS**

In the study were included 103 patients: 25 men and 78 women in the age range of 28 to 81 years, with median age of 55.8 years (SD±11.8). According to endoscopic results patients were classified into three groups: 68 (66%) with non-ulcer dyspepsia (NUD), 22 (21.4%) with erosio/ulcus ventriculi (EUV), and 13 (12.6%) with erosio/ulcus duodeni (EUD).

Out of 103 *H. pylori* isolates 16 (15.5%) had deleted *cag*PAI, and 87 (84.5%) partially deleted *cag*PAI. None of the isolates had intact *cag*PAI. There was no statistically significant difference in the distribution of *cag*PAI either according to the gender (χ²=0.005, df=1, p=0.941) or according to the endoscopic diagnosis (χ²=1.142 df=2, p=0.565) as shown in Table 2.

The frequency of particular genes was as follows: *cagA1* 71.8%, *cagA2* 69.9%, *cagA3* 5.8%, *cagE* 75.7%, *cagM* 71.8%, *tnpA* 9.7%, *cagT* 68%, *Apcag* 63.1%, *LEC* 48.5% and *tnpB* 7.8% as shown in Figure 1.

**Table 1. Primers used in this study**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Primer sequence</th>
<th>Amplicon size</th>
<th>Annealing temp.</th>
<th>Reference</th>
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</thead>
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<tr>
<td><em>cagM</em></td>
<td>Cag 5</td>
<td>ACAAATACAAAAAAGAAAAAGAGGC</td>
<td>586 bp</td>
<td>53 °C</td>
<td>10</td>
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<td></td>
<td>Cag 6</td>
<td>ATTTTTCAACAGTTAGAAAAAGCC</td>
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<tr>
<td><em>tnpA</em></td>
<td>Cag10</td>
<td>ATCGTGCTAAAAAGTTTTTCTTTC</td>
<td>344 bp</td>
<td>53 °C</td>
<td>10</td>
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<td></td>
<td>Cag11</td>
<td>TAAGGAGGTATATTCAACAAACGG</td>
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<tr>
<td><em>tnpB</em></td>
<td>Cag 8</td>
<td>ACAATATACAAAAAGAAAAAGAGGC</td>
<td>569 bp</td>
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<td>10</td>
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<td></td>
<td>Cag 9</td>
<td>AGCTAGGGAAAAATCTGTCTATG</td>
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<td><em>cagA2</em></td>
<td>CAG 1</td>
<td>AGACACAGTCGCGAGAAAAAGG</td>
<td>320 bp</td>
<td>53 °C</td>
<td>11</td>
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<td>TATTGGATTCTTGAGGCCC</td>
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<td><em>cagE</em></td>
<td>CagE-F1</td>
<td>ACAAATACAAAAAGAAAAAGAGGC</td>
<td>329 bp</td>
<td>52 °C</td>
<td>12</td>
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<td><em>cagT</em></td>
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<td><em>cagA3</em></td>
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<td>AACAGGACAAGATGCTACTGCC</td>
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<tr>
<td><em>cagA1</em></td>
<td>CagA-F2</td>
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<td>349 bp</td>
<td>52 °C</td>
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<td><em>Apcag</em></td>
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<td>730 bp</td>
<td>52 °C</td>
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<td>AP-F1</td>
<td>GTGGGTAAAAGATTGAATTCG</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>LEC</em></td>
<td>LEC-F1</td>
<td>ACATTTTGCTAAATAAACGCT</td>
<td>320-550 bp</td>
<td>55 °C</td>
<td>13</td>
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<td></td>
</tr>
<tr>
<td><em>Ag</em></td>
<td>P1</td>
<td>TGGCGTTGTCTATTGACGCGAC</td>
<td>298 bp</td>
<td>57 °C</td>
<td>14</td>
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<tr>
<td></td>
<td>P2</td>
<td>CTGCGTGGGGACTATTCCATCAGT</td>
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</table>
Pathohistological analysis of antrum revealed inflammation in all patients (100%), activity was found in 66% of the patients, atrophy in 6.8% and intestinal metaplasia in 24.3%. All patients had inflammation in the corpus. Activity was found in 62.2% of the patients. Atrophy was present in 4.9% and metaplasia in 13.6% of the patients. Table 3. shows the distribution of different grades of pathohistological parameters of gastritis in the antrum and corpus.

There was no statistically significant difference in pathohistological lesions between the patients with partially deleted cagPAI and those with deleted cagPAI (p>0.05 for all parameters describing pathohistological lesions).

The presence of CagA2 was significantly related to the higher grade of inflammation of antrum ($\chi^2=6.872, df=2, p=0.032$), with increased density of H. pylori in the corpus ($\chi^2=16.7, df=3, p=0.001$), and with higher total score for the corpus (mean±SD=4.1±1.5 for CagA2+, 3.3±1.4 for CagA2−, t=2.687, p=0.008) as shown in Table 4.

The presence of Apaccag was significantly correlated with higher inflammatory score of antrum (Apaccag+:mean±SD=4.9±1.7; Apaccag−: 4.0±1.7; t=2.332, p=0.022) (Table 4).

The presence of cagM was related to the higher density of H. pylori in the corpus ($\chi^2=9.864, df=3, p=0.020$), and higher total score for the corpus (CagM+: mean±SD=4.1±1.6; CagM−: 3.4±1.4; t=2.021, p=0.046) (Table 4).

The presence of cagT and LEC was related to less frequency of antrum atrophy (cagT, $\chi^2=5.35, df=1, p=0.021$).

Correlation between any of ten cagPAI genes and endoscopic diagnosis (p>0.16 for all) was not found in this study.

**DISCUSSION**

In our study, we amplified 10 H. pylori genes in order to characterize cagPAI. Intact cagPAI was not found but there was 84.5% partially deleted, and 15.5% completely deleted cagPAI genes from H. pylori isolates (N=103)
deleated. In contrast, in the study done in Mexico which included 11 genes, there was 90% of intact cagPAI, 4% of partially deleated and 6% of completely deleated (16).

We want to emphasise that there is disconcordance between different studies in the number of cagPAI genes analysed, and the definition of intact, deleated and partially deleated cagPAI. Most studies analysed limited number of genes. Salih et al. analyzed 4 genes of cagPAI and reported 42.1% of intact, 39.5% of partially deleated and 18.4% completely deleated cagPAI and the correlation of intact cagPAI and duodenal ulcer (17). Baghaei et al. analyzed three genes and reported 17% of intact cagPAI, 62% of partially deleated and 20% of completely deleated in Iran population (8). Nygen et al. analysed 30 genes with the same number of strains and similar endoscopic diagnosis as in our study and found 88% of intact, and 12% of partially deleated in Vietnam population (18). Based on bibliographical data it is evident that the frequency of intact cagPAI varies depending on the geographic area.

Results of our study did not demonstrate any correlation between deleated and partially deleated cagPAI and either endoscopic diagnosis or pathohistological lesions. It is hard to explain wheter our results reflect the situation in our geographic region or if they are related to a specific category of patients with predominant non-ulcer dyspepsia. The study from Maeda et al. from Japan confirmed our observation that partially deleated cagPAI is associated with non-ulcer dyspepsia in contrast with intact cagPAI found in patients with gastric cancer (19). We do not have the data for different categories of patients with other grades of gastroduodenal disease. This is the first study of genotyping of cagPAI in Croatia. The future studies should be focused on genotyping of cagPAI in Croatian patients with severe gastroduodenal disease.

CagA is considered to be a marker of cagPAI region (20). In our study we analysed three different segment of cagA gene. cagA1 segment close to the promoter region, middle segment cagA2 and right end cagA3. While the rate of cagA1 and cagA2 positivity was similar (71.8%) and (69.9%) respectively, the frequency of cagA3 was low (5.8%). The frequent deletion of cagA3 compared to cagA1 and cagA2 in the control strains reported by Matar et al. was attributed to decreases pathogenicity (9). Prevalence of cagA positive strains differs between the countries and is the highest in East Asia (90%), Japan (100%) (21) and Bulgaria (84.9%) (22). The moderate prevalence was found in Iran (62%) (23), Slovenia (61%) (24), Columbia (64%) (21), Turkey (49%) (25), Equador (46%) (26) and Portugal (31.8%) (27). The previous studies on H. pylori in Croatia reported the prevalence of serum antibodies

<table>
<thead>
<tr>
<th>Pathohistology</th>
<th>Grade</th>
<th>Negative</th>
<th>Positive</th>
<th>Statistics</th>
<th>P-value</th>
<th>OR (95% CI)</th>
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<tr>
<td><strong>cagA2</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Inflammation, antrum</td>
<td>1</td>
<td>16</td>
<td>19</td>
<td>χ²=6.872</td>
<td>0.032</td>
<td>2.941 (1.216–7.217) for grade 1 vs. 2/3</td>
</tr>
<tr>
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<td>15</td>
<td>50</td>
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</tr>
<tr>
<td></td>
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<td>1</td>
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<tr>
<td>Total score, corpus</td>
<td>Mean±SD</td>
<td>3.3±1.4</td>
<td>4±1.5</td>
<td>t=2.687</td>
<td>0.008</td>
<td>3.841 (1.531–9.638)</td>
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<tr>
<td></td>
<td>1</td>
<td>24</td>
<td>37</td>
<td>χ²=9.864</td>
<td>0.020</td>
<td>8.665 (2.621–38.73) for grade 0/1 vs. 2/3</td>
</tr>
<tr>
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<tr>
<td>Total score, corpus</td>
<td>Mean±SD</td>
<td>3.4±1.4</td>
<td>4±1.6</td>
<td>t=2.021</td>
<td>0.046</td>
<td>3.259 (1.293–8.215)</td>
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<td>Atrophy, antrum</td>
<td>0</td>
<td>28</td>
<td>68</td>
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<tr>
<td></td>
<td>1</td>
<td>5</td>
<td>2</td>
<td>χ²=5.352</td>
<td>0.021</td>
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<td>Inflammatory score, antrum</td>
<td>Mean±SD</td>
<td>2.2±1.0</td>
<td>2.7±1.1</td>
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<td>Total score, antrum</td>
<td>Mean±SD</td>
<td>4.0±1.7</td>
<td>4.9±1.7</td>
<td>t=2.332</td>
<td>0.022</td>
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<td>50</td>
<td></td>
<td>0.016*</td>
<td>0 (0–0.524)</td>
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</tbody>
</table>
against bacterial virulence antigens p120 (CagA- cytotoxin associated antigen) of 91.3% in the group of patients with severe gastroduodenal diseases (28). In our study the prevalence of cagA was not statistically significantly correlated with clinical diagnosis which is in concordance with the study of Strauss et al. (81% positive) (29) but different from the study of Marie M et al. (62% positive) where the presence of cagA was correlated with gastritis and peptic ulcer (30). In our study the presence of cagA was correlated with higher degree of inflammation in the gastric mucosa, particularly in antrum (p=0.001). In the previous study from Croatia p120 (cagA) seropositivity was significantly more often present in patients with higher activity grade in the antrum (28). These results are in concordance with other studies which proved that cagA enhances accumulation of neutrophiles, determined as inflammatory score and according to some studies induces the production of interleukine IL-8 (17). These results are confirmed by studies from Iran (31). No correlation between cagA and cagE and clinical outcome in Iran patients was found (32). In our study cagA2 is related to the higher density of H. pylori (p=0.001) and this correlation was confirmed by other authors (33–35). However, some studies did not find any significant relationship between cagA positivity and H. pylori density, neutrophil activity, lymphoid aggregation in lamina propria and glandular atrophy in the biopsies, but significant association was detected with severe chronic gastritis (23). The frequency of cagE in our study was higher than of cagA (75.7% vs 71.8%). This is in agreement with some studies which consider cagE to be a better marker of cagI region than cagA (9, 36). We did not find any association between cagE and endoscopic diagnosis and pathohistological lesions which is in agreement with the results from a study conducted in Portugal where cagE is more prevalent than cagA (27). Modena at al. have not found association between cagE and clinical outcome (37), contrarily to the studies which described higher frequency of cagE and sever gastroduodenal disease such as ulcer and gastric cancer, than in gastritis (3). CagT as a marker of cagII region was identified in 68% of our isolates and was associated with decreased frequency of antrum atrophy without any correlation with clinical diagnosis. However, some authors did not report correlation with either clinical diagnosis or pathohistological alterations of gastric mucosa (8). Mattar et al. reported that 98% of the patients with ulcer disease retained cagT gene (9), while the isolates with deleted cagT were more frequent in the patients with chronic gastritis compared with peptic ulcer disease or gastric cancer in Japanese population (12). Fisher et al. claim that the patients with H. pylori lacking cagT have disfunctional T4SS and are unable to translocate cagA protein into the host cell (38). In the study from England the majority of ulcer disease strains retained the cagT and cagE gene (39). In our study cagM with the prevalence of 71.8% was associated with increased density of H. pylori in corpus and higher total score for corpus, but unrelated to the endoscopic diagnosis. Matar et al. correlated this gene with higher grade of gastritis and peptic ulcer disease (9). LEC (left end of cagII) was found in 48.5% of our isolates and was related to the lower prevalence of antrum atrophy. The LEC is rearranged more frequently in isolates linked to severe pathology (40).

This study comprised the patients without successful eradication of H. pylori infection after multiple antibiotic courses in spite of the fact that antimicrobial therapy was after one or two unsuccessful therapeutic outcomes created in accordance with antimicrobial susceptibility testing.

The most patients had normal or harmless endoscopic result (non-ulcer dispepsia). Although pathological alterations did not point out to the danger of premalignant lesions our study found a high frequency of cagA, ApCag, cagT and cagM genes in the isolates recovered from the patients included in the study. The correlation between the presence of these genes and higher degree of serious pathohistological lesions in gastric mucosa was observed. According the the results of the present study it could be concluded that the presence of these genes can predispose for the development of ulcer, premalignant or malignant diseases. Thus, insisting on eradication of H. pylori in spite of harmless endoscopy and histological results should be considered as the only correct choice.

In spite of the fact that application of molecular diagnostics in detection of virulence genes is too expensive and not recommended for routine diagnostic, it should have a role in selected patients with unsuccessful eradication therapy with usual therapeutic protocols.

Moreover, the genomic profiles generated in this study may be useful for interlaboratory comparisons and are suitable for storage in epidemiological databases for comparative analyses. Our study has been focused on a specific group of patients isolates and may be representative for isolates from patients in this geographic region in Croatia. Future studies are needed to involve other disease specific strain group with appropriate controls.

CONFLICT OF INTEREST

There is no conflict of interest

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