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original research article

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

DIJANA VARDA BRKIĆ[1]
MIROSLAVA KATIČIĆ[2,3]
BRANKA BEDENIĆ[1,3]
ALEKSANDRA PRESEČKI STANKO[1]
VANDA PLEČKO[1,3]

[1] Department of Clinical and Molecular Microbiology, University Hospital Centre Zagreb, Kišpatićeva 12, Zagreb

[2] University Hospital Merkur, Zajčeva 19, Zagreb

[3] School of Medicine, University of Zagreb, Zagreb, Croatia

Correspondence:
Dijana Varda Brkić
dijanavb098@gmail.com

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Abstract

Background: *Some of the genes belonging to cag pathogenicity island (cag*PAI*) 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* cag*PAI island and comparison with pathohistological 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* cag*PAI: Ap*cag *(*cagA *promotor region),* cagA1*,* cagA2*,* cagA3*,* cagM, cagT, cagE, LEC, tnpA *and* tnpB*. 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 parcially deleated* cag*PAI. None of the isolates possessed all 10 genes. The frequency of single* cag*PAI genes were as follows: Ap*cag *63.1%,* cagA1 *71.8%,* cagA2 *69.9%,* cagA3 *5.8%,* cagM *71.8%,* cagE *75,7%, cagT 68%,* tnpA *9.7%,* tnpB *7.8% i* LEC *48.5%.*

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

Conclusions: H. pylori *isolates with positive* cagA*, Ap*cag *and* cagM *genes are correlated to higher degree of patohistological lesions of gastric mucosa; without statistically significant correlation with endoscopic diagnosis.*

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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 (*cag*PAI) *(1). Cag*PAI is since its discovery the most analysed

segment of *H. pylori* genome. It is approximately 40 kilobase pairs region of *H. pylori* chromosome and contains around 30 genes divided into region I and II. *Cag*PAI is defined as intact if all genes are present, parcially deleated as presence of several genes and negative (deleated) if there are no genes at all (2). Many studies report correlation between presence of intact *cag*PAI, partially deleated and deleated *cag*PAI and clinical outcome. Some authors correlate intact *cag*PAI with severe gastroduodenal diseases, with higher grade of chronic gastritis and premalignant lesions of gastric mucosa. *(1, 3–4)*, while partially deleated and deleated *cag*PAI are associated with milder forms of gastroduodenal disease and lower grade of pathological alternations of gastric mucosa *(5–6)*. On the contrary, some of the authors did not find correlation between *cag*PAI and gastroduodenal disease *(7)*. *Cag*PAI encodes multiple structural components of bacterial type IV secretion system (T4SS). T4SS translocates cagA protein directly to the cytosol of the gastric epitelium where it gets tyrosin phosphorylated by Src-family kinases and becomes able to alter the host cell functions leading to malignant transformation *(2)*.

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

CagE is also located in the region I and is necessary to induce production of interleukin IL-8. Some authors consider *cagE* gene to be better marker of *cag*PAI region compared to *cagA* and more useful in monitoring the progress of *H. pylori* induced gastric disease. *CagT* gene is a marker of cagII region and some studies connect it with more severe clinical disease *(8)*. *LEC* (left terminal end of cagII) is not necessary for translocation of cagA into the host cell or induction of interleukin IL8. It is associated with peptic ulcer and adenocarcinoma. Some of the study found connection of *tnpA* gene with peptic ulcer *(9)*.

There are no published studies on the presence of *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 *H. pylori* just in these patients as a possible predictors of future severe gastroduodenal diseases, by comparing it with clinical and pathohistological results.

MATERIAL AND METHODS

Patients

The study analysed gastroscopic test results and bioptic specimens of gastric mucosa with positive *H. pylori* culture in 103 patients examined during routine, clinical gastroduodenoscopies in the endoscopic laboratory of the University Hospita 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 specimens at the Department for Pathology of University Hospital Merkur. The study was approved by the Ethical Comittee 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 *H. pylori*. According to the endoscopic finding patients were classified in three groups: non ulcer dyspepsia (NUD), erosio/ulcus ventriculi (EUV), erosio/ulcus duodeni (EUD).

Bacterial culture

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

DNA extraction

Extraction of chromosomal DNA was performed with commericial kit: High Pure PCR Template Preparation Kit, Version 16 (Roche Diagnostics Gmbh, Mannheim, Germany) according to the manufacturer's recommendation. The DNA was stored at –20°C until used for molecular studies.

PCR amplification

PCR was used to detect the following genes: *cagA1*, *cagA2*, *cagA3*, *cagE*, *cagM, cagT*, *cagA* promotor region (Ap-*cag*), *tnpA*, *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 *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 visualised by electrophoresis in in 2% agarose gel, after staining with ethidium bromide and examined in UV transilluminator. A 100 bp DNA ladder (Sigma) was used as a size marker. Reference strains 47164 and 17874 (Culture collection, University of Goethenburg) were used a positive control.

Histology

Specimens for pathohistological analysis were fixed in a standard 4% neutral buffered formalin, and cut into

Table 1. *Primers used in this study*

Gene	Primer	Primer sequence	Amplicon size	Annealing temp.	Reference
cagM	Cag 5	ACAAATACAAAAAAGAAAAAGAGGC	586 bp	53 °C	10
	Cag 6	ATTTTTCAACAAGTTAGAAAAAGCC			
tnpA	Cag10	ATCAGTCCAAAAAGTTTTTTCTTTCC	344 bp	53 °C	10
	Cag11	TAAGGGGGTATATTTCAACCAACCG			
tnpB	Cag 8	ACAAATACAAAAAAGAAAAAGAGGC	569 bp	53 °C	10
	Cag 9	AGCTAGGGAAAAATCTGTCTATGCC			
cagA2	CAG1	AGACAACTTGAGCGAGAAAG	320 bp	53 °C	11
	CAG-2	TATTGGGATTCTTGGAGGCG			
cagE	CagE-F1	ACAAATACAAAAAAGAAAAAGAGGC	329 bp	52 °C	12
	CagE-R1	GAAGTGGTTAAAAAATCAATGCCCC			
cagT	CagT-F1	CCATGTTTATACGCCTGTGT	301bp	52 °C	12
	CagT-R1	CATCACCACACCCTTTTGAT			
cagA3	CagA-F1	AACAGGACAAGTAGCTAGCC	701 bp	52 °C	12
	CagA-R1	TATTAATGCGTGTGTGGCTG			
cagA1	CagA-F2	ACAAATACAAAAAAGAAAAAGAGGC	349 bp	52 °C	12
	CagA-R2	CTGCAAAAGATTGTTTGGCAGA			
Ap*cag*	CagA-R2	CTGCAAAAGATTGTTTGGCAGA	730 bp	52 °C	12
	AP-F1	GTGGGTAAAAATGTGAATCG			
LEC	LEC-F1	ACATTTTGGCTAAATAAACGCTG	320-550 bp	55 °C	13
	LEC-R1	TCTCCATGTTGCCATTATGCT			
Ag	P1	TGGCGTGTCTATTGACAGCGAGC	298 bp	57 °C	14
	P2	CGTGCTGGGCATACTTCACCATG			

slides. Slides were routinely stained ana 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 χ test or Fischer exact test with the calculation of odds ration (OR) together with 95% confidence intervals (CI). Logistic regression analysis was used to calculate OR (95%CIs) 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 woman 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 deleated *cag*PAI, and 87 (84.5%) partially deleated *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 th 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%, Ap*cag* 63.1%, *LEC* 48.5% and *tnpB* 7.8% as shown in Figure 1.

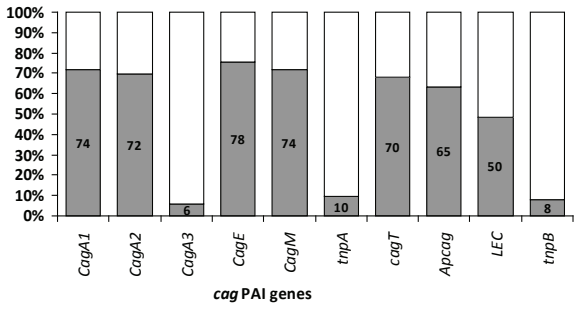

Figure 1. *Percentage and number of positive cagPAI genes from H. pylori isolates (N=103)*

Table 2. *Correlation of cagPAI and endoscopic diagnosis*

Endoscopic diagnosis	Partialy deleted *cag*PAI	Completely deleted *cag*PAI
EUV 22 (21.4%)	17 (77.3%)	5 (22.7%)
EUD 13 (12.6%)	11 (84.6%)	2 (15.4%)
NUD 68 (66.0%)	59 (86.8%)	9 (13.2%)

EUV – erosio/ulcus ventriculi; **EUD** – erosio/ulcus duodeni; **NUD** – non-ulcer dyspepsia.

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 parcially deleated *cag*PAI and those with deleated *cag*PAI (p>0.05 for all parameters describing pathohistological lesions).

The presence of *CagA2* was significantly related to the higher grade of inflammation of antrum (χ2=6.872, df=2, p=0.032), with increased density of *H. pylori* in the corpus (χ2=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 Ap*cag* was significantly correlated with higher inflammatory score of antrum (Ap*cag*+:mean± SD=4.9±1.7; Ap*cag*-: 4.0±1.7; t=2.332, p=0.022) (Table 4).

The presence of *cagM* was related to the higher densitiy of *H. pylori* in the corpus (χ2=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).

Table 3. *Pathohistological characteristics of the patients (n=103)**

	Characteristics	Positivity	Score	No (%)	Mean (SD)
Antrum	Activity PMN	68 (66%)	0 1 2 3	35 (34.0) 50 (48.5) 15 (14.6) 3 (2.9)	
	Inflammation	103 (100%)	1 2 3	35 (34.0) 65 (63.1) 3 (2.9)	
	Inflammatory score				2.5 (1.1)
	Atrophy	7 (6.8%)	0 1	96 (93.2) 7 (6.8)	
	Metaplasia	25 (24.3%)	0 1	78 (75.7) 25 (24.3)	
	H. pylori	92 (89,3%)	0 1 2 3	11 (10.7) 33 (32.0) 34 (33.0) 25 (24.3)	
	Total score				4.5 (1.8)
Corpus	Activity PMN	64 (62.2%)	0 1 2	39 (37.9) 53 (51.5) 11 (10.7)	
	Inflammation	103 (100%)	1 2 3	55 (53.4) 43 (41.7) 5 (4.9)	
	Inflammatory score				2.2 (1.0)
	Atrophy	5 (4,9%)	0 1 2	98 (95.1) 4 (3.9) 1 (1.0)	
	Metaplasia	14 (13,6%)	0 1	89 (86.4) 14 (13.6)	
	H. pylori	99 (96,1%)	0 1 2 3	4 (3.9) 61 (59.2) 25 (24.3) 13 (12.6)	
	Total score				3.8 (1.5)
Total score antrum+corpus					8.4 (2.5)

*Accoridng to the Updated Sydney system classification with metaplasia absent=0 and present=1, PMN= Polymorph-nuclear cells

The presence of *cagT* and *LEC* was related to less frequency of antrum atrophy *(cagT,* χ2=5.35, df=1, p=0.021).

Correlation between any of ten *cag*PAI 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 *cag*PAI. Intact *cag*PAI was not found but there was 84.5% parcially deleated, and 15.5% completely

Table 4. *Association of H. pylori cagPAI genes with pathohistological changes of gastric mucosa*

Pathohistology						
	Grade	Negative	Positive	Statistics	P-value	OR (95% CI)
cagA2						
Inflammation, antrum	1 2 3	16 15 0	19 50 3	$\chi2$=6.872	0.032	2.941 (1.216–7.217) for grade 1 vs. 2/3
H. pylori, corpus	0 1 2 3	3 25 1 2	1 36 24 11	$\chi2$=16.700	0.001	15.02 (4.558–67.12) for grade 0/1 vs. 2/3
Total score, corpus	Mean±SD	3.3±1.4	4.1±1.5	t=2.687	0.008	3.841 (1.531–9.638)
CagM						
H. pylori, corpus	0 1 2 3	1 24 2 2	3 37 23 11	$\chi2$=9.864	0.020	8.665 (2.621–38.73) for grade 0/1 vs. 2/3
Total score, corpus	Mean±SD	3.4±1.4	4.1±1.6	t=2.021	0.046	3.259 (1.293–8.215)
cagT						
Atrophy, antrum	0 1	28 5	68 2	$\chi2$=5.352	0.021	0.165 (0.030–0.918)
Ap*cag*						
Inflammatory score, antrum	Mean±SD	2.2±1.0	2.7±1.1	t=2.283	0.025	2.448 (1.285–26.008)
Total score, antrum	Mean±SD	4.0±1.7	4.9±1.7	t=2.332	0.022	2.349 (1.027–5.374)
LEC						
Atrophy, antrum	0 1	46 7	50 0		0.016*	0 (0–0.524)

deleated. In contrast, in the study done in Mexico which included 11 genes, there was 90% of intact *cag*PAI, 4% of parcially delated and 6% of completely deleated *(16).*

We want to emphasise that there is disconcordance between different studies in the number of *cag*PAI genes analysed, and the definition of intact, deleated and parcially deleated *cag*PAI. Most studies analysed limited number of genes. Salih et al. analyzed 4 genes of *cag*PAI and reported 42.1% of intact, 39.5% of partially deleated and 18.4% completely deleated *cag*PAI and the correlation of intact *cag*PAI and duodenal ulcer (17). Baghaei et al. analyzed three genes and reported 17% of intact *cag*PAI, 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 parcially deleated in Vietnam population *(18).* Based on bibliographical data it is evident that the frequency of intact *cag*PAI varies depending on the geographic area.

Results of our study did not demonstrate any correlation between deleated and partially deleated *cag*PAI 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 *cag*PAI is associated with non-ulcer dyspepsia in contrast with intact *cag*PAI 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 *cag*PAI in Croatia. The future studies should be focused on genotyping of *cag*PAI in Croatian patients with severe gastroduodenal disease.

CagA is considered to be a marker of *cag*PAI 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

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 *cag*I 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 successfull 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*, Ap*Cag*, *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 predispone for the development of ulcer, premalignant or malignant diseases. Thus, insisting on eradication of *H. pylori* in spite of harmless endoscopis 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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