

# *Helicobacter* Genotyping and Detection in Peroperative Lavage Fluid in Patients with Perforated Peptic Ulcer

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Received: 6 July 2007 / Accepted: 12 August 2007 / Published online: 29 September 2007  
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## Abstract

**Introduction and Objectives** Certain *Helicobacter pylori* genotypes are associated with peptic ulcer disease; however, little is known about associations between the *H. pylori* genotype and perforated peptic ulcer (PPU). The primary aim of this study was to evaluate which genotypes are present in patients with PPU and which genotype is dominant in this population. The secondary aim was to study the possibility of determining the *H. pylori* status in a way other than by biopsy.

**Materials and Methods** Serum samples, gastric tissue biopsies, lavage fluid, and fluid from the nasogastric tube were collected from patients operated upon for PPU. By means of PCR, DEIA, and LIPA the presence of the “cytotoxin associated gene” (*cagA*) and the genotype of the “vacuolating cytotoxin gene” were determined.

**Results** Fluid from the nasogastric tube was obtained from 25 patients, lavage fluid from 26 patients, serum samples from 20 patients and biopsies from 18 patients. Several genotypes were found, of which the *vacA* s1 *cagA* positive strains were predominant. Additionally, a correlation was found between the *H. pylori* presence in biopsy and its presence in lavage fluid ( $p=0.015$ ), rendering the latter as an alternative for biopsy. Sensitivity and specificity of lavage fluid analysis were 100% and 67%, respectively.

**Conclusion** This study shows the *vacA* s1 *cagA* positive strain is predominant in a PPU population. The correlation found between the *H. pylori* presence in biopsy and its presence in lavage fluid suggests that analysis of the lavage fluid is sufficient to determine the *H. pylori* presence. Risks associated with biopsy taking may be avoided.

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This work was presented at the 42nd Congress of the European Society for Surgical Research 2007, Rotterdam, The Netherlands, May 26<sup>th</sup>, 2007.

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**Keywords** *H. pylori* · Perforated Peptic Ulcer · Genotype · Peroperative lavage fluid · Peptic Ulcer Disease

## Introduction

Over the past decades the incidence of perforated peptic ulcer (PPU) has declined in the western world. However, with an incidence varying between two and 10 per 100,000, it still is a problem in modern society.<sup>1</sup> Moreover, mortality rates caused by gastric and duodenal ulcer perforation vary between 10 and 40% and zero and 10% respectively, and is higher among elderly patients.<sup>2,3</sup> Several risk factors for PPU have been described such as smoking, alcohol abuse, and history of peptic ulcer disease (PUD).<sup>2</sup> However, the main pathogenic factors are considered to be the use of non-steroidal anti-inflammatory drugs (NSAID) and the presence of *H. pylori*.<sup>2</sup>

*Helicobacter pylori* are widespread bacteria, with a prevalence ranging from 25% in the industrialized world to more than 70% in developing countries.<sup>4,5</sup> Most infected people remain asymptomatic; however, a small group of carriers will develop PUD.

Of patients who have developed PPU, 70% will test positive for *H. pylori*,<sup>2</sup> suggesting the pathogenesis of perforation is associated with the presence of *H. pylori*. In addition, it is shown that different genotypes of *H. pylori* are associated with different clinical manifestations like PUD and gastric cancer.<sup>6,7</sup> Two well-known *H. pylori* genes that have been associated with PUD are the cytotoxin-associated gene (*cagA*) and the vacuolating cytotoxin gene (*vacA*).<sup>6,8–10</sup>

*VacA* is present in all *H. pylori* strains and is associated with gastritis, PUD, and gastric carcinoma.<sup>10–12</sup> It encodes for a vacuolating cytotoxin that causes epithelial cell injury and interferes with the immune system.<sup>13,14</sup> *VacA* contains at least two variable regions, the signal peptide (s)-region and the middle (m)-region. The s-region contains two allelic types, s1 and s2. The s1 strain has several subtypes, being s1a, s1b, and s1c.<sup>15</sup> Two allelic types exist for the m-region, m1 and m2. The latter has two subtypes, m2a and m2b.<sup>16</sup>

*CagA* is considered a marker for a genomic pathogenicity (*cag*) island that is associated with enhanced virulence.<sup>17</sup>

If PPU is associated with a specific *H. pylori* genotype it may be feasible to limit the patients undergoing antibiotic therapy to those who have this genotype. When this specific type is not present, another cause of PPU should be looked for and antibiotic therapy should not be started. This would mean cost reduction and, probably, a reduction in the speed of the development of antibiotic resistance.

Currently, gastric biopsy during endoscopy is a generally accepted method to diagnose *H. pylori* infection. However, patients with PPU will not undergo endoscopy but will generally be operated upon immediately. Taking a biopsy intraoperatively implicates a higher risk of bleeding and more difficult closure of the defect. Therefore, surgeons are reluctant to take a biopsy.

The primary aim of this study was to evaluate which genotypes are present in patients with PPU and if a genotype is dominant in this population. The secondary aim was to study the possibility of determining the *H. pylori* status in a manner other than by gastric tissue biopsy.

## Methods

From 30 consecutive patients operated on for PPU serum samples, gastric tissue biopsies, lavage fluid, and fluid from the nasogastric tube were collected. These patients were treated in five different medical centers throughout the

Netherlands. In each of these centers approval of the medical ethical committee was obtained. Immediately after collection, the materials were frozen at  $-20^{\circ}\text{C}$ . One researcher performed the analysis and genotyping. For *H. pylori* genotyping, the presence of cytotoxin-associated gene (*cagA*) and the s- and m-region genotypes of the vacuolating cytotoxin gene (*vacA*) were determined.

DNA was isolated according to Boom's method as described previously.<sup>18</sup> A guanidine thiocyanate (GuSCN) solution was added to the collected material to induce lysis of the bacteria, releasing their DNA. After addition of the silica particles (Celite) the suspension was centrifuged. The silica particles, with the attached DNA, were washed with subsequently GuSCN-containing washing buffer, ethanol 70% and acetone. After drying, the DNA was eluted in an aqueous low salt buffer. The isolated DNA was amplified by means of polymerase chain reaction (PCR) and subsequently the presence of *cagA* and different types of *vacA* were analyzed by means of reverse hybridization on a strip (32). This assay consists of a nitrocellulose strip that contains dT-tailed oligonucleotide probes immobilized as parallel lines. For each strain, 10  $\mu\text{l}$  of each PCR product (containing biotin at the 5' end of each primer) was denatured by the addition of an equal amount of 400 mM NaOH and 10 mM EDTA in a plastic trough. After 5 min, 1 ml of prewarmed hybridization solution (2 $\times$  SSC [1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 50 mM Tris-HCl [pH 7.5], 0.1% SDS) was added, and a strip was submerged and incubated in a shaking water bath at 50 $^{\circ}\text{C}$  for 1 h. The strips were washed with 2 ml of 2 $\times$  SSC-0.1% SDS for 30 min at 50 $^{\circ}\text{C}$ . Subsequently, the strips were rinsed three times in phosphate buffer, and conjugate (streptavidin-alkaline phosphatase) was added. After incubation at room temperature for 30 min, the strips were rinsed again and 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate substrate was added. Hybrids are visible as purple probe lines. Interpretation of the hybridization patterns was performed visually. As a control, a  $\beta$ -globin PCR was performed. Patient related factors were obtained prospectively. Statistical analysis was performed with SPSS for Windows, version 11.0.

## Results

A total of 30 patients were included of whom nine were women. The average age was 65 years, varying between 40 and 87. Ten patients (33.3%) were operated laparoscopically. The perforation was found prepyloric in 11 patients, at the site of the pylorus in eight patients and postpyloric in 11 patients.

A total of five (16.7%) patients had a history of PUD. Ten patients (33.3%) used NSAID's, two patients (6.7%)

**Table 1** *Helicobacter pylori* Status and Genotype

Patient	Fluid from naso-gastric tube	Lavage fluid	Serum	Gastric tissue biopsy
1	s1a/m2a/cag			
2	s1a/m2a/cag	s1a/m2a/cag		s1a/m2a/cag
3	s1b	s1a/m2a/cag		
4		s1a/s2/m2a		
5		s1a		s1a/m1
6				
7	s1a/m2a			
8				
9	s1b/m1/cag	s1b/m1/cag		
10				
11				
12		s1a/m2a/cag		
13				
14	s1a/m1/cag			s1a/m1/cag
15		s1a/m1/cag		s1a
16	s1a/m1/cag	s1a/m1/cag		
17	s1a/m1/cag			
18				
19	s1a/m1/cag	s1a/m1/cag		
20	s1a/m1/cag	s1a/m1/cag		
21	s1a/m1/cag	s1a/m1/cag		
22				
23		s1a/m1/cag		
24	s1a/s1b/m1/cag	s1a/s1b/m1/cag		s1b/m1
25		s1a/m1/cag		s1b/m1/cag
26		s1a/m2a/cag		
27		s1a/m2a		s1a/m1/cag
28	s1a/s2/m2a/cag	s1a/s2/m2a/cag		s1a/s2/m2a/cag
29		s2/m2a		s2/m2a
30				
	β-globin and <i>H. pylori</i> positive			
	β-globin and <i>H. pylori</i> negative			
	β-globin positive and <i>H. pylori</i> negative			
	β-globin negative and <i>H. pylori</i> positive			
	No materials			

The colors represent the β-globin and *H. pylori* status of the patient.

used steroids, three patients (10.0%) used acid reducers, and one patient (3.3%) used a proton pump inhibitor (PPI) before admission to the hospital. The average hospital stay was 11.9 days, varying between 3 and 37 days.

Fluid from the nasogastric tube was obtained from 25 patients, lavage fluid from 26 patients, serum samples from 20 patients, and ulcer biopsies from 18 patients. The results of the genotyping are depicted in Table 1.

The β-globin determination was performed as a control. In nine samples of nasogastric tube fluid and in two samples of lavage fluid it was negative, rendering these

results as unreliable. Therefore, these results were excluded from further analysis.

Table 2 represents the frequency of the individual genes and the allelic types found in the different samples by means of PCR and LiPA.

These tables show that for *vacA* the allelic type s1 is predominantly present in all three types of samples. In the s1 positive strains, subtype s1a is predominant as depicted in Table 3.

With regard to the middle region of *vacA* the incidence of m1 allelic type is slightly higher; however, the difference

**Table 2** Frequencies of Individual Genes and Allelic Types

Genotype	Fluid from Naso-Gastric Tube		Lavage Fluid		Gastric Tissue Biopsy		Control Non-Ulcer
	No.	%	No.	%	No.	%	%
<i>VacA</i> s1	10	90.9	14	77.8	7	77.8	46.9
<i>VacA</i> s2	0	0	1	5.5	1	11.1	38.4
<i>VacA</i> multiple	1	9.1	3	16.7	1	11.1	14.7
Total	11	100	18	100	9	100	100
<i>VacA</i> m1	6	54.5	9	50.0	5	55.6	29.4
<i>VacA</i> m2	4	36.4	8	44.4	3	33.3	55.9
<i>VacA</i> incomplete genotype	1	9.1	1	5.6	1	11.1	0 (14.7 % incomplete)
Total	11	100	18	100	9	100	100
<i>CagA</i> positive	9	81.8	14	77.8	5	55.6	47.1
Total	11	100	18	100	9	100	100

“*VacA* multiple” means that more than one allelic type or subtype has been found in one sample.

In each different type of sample one incomplete genotype occurred, which is indicated as “*vacA* incomplete”. The “Control non ulcer” column represents the frequencies, found by van Doorn et al., in a population without PUD and is added to allow easy comparison.

is less outspoken compared to s1. The m2a was the only subtype that was found in the samples. In three samples, the genotyping was incomplete (Tables 1 and 2), meaning that determination of the middle region was not possible. This was most likely caused by the small number of bacteria present in those samples.

With regard to the secondary aim of this study, analyzing possibilities to diagnose *H. pylori* presence in another fashion than through biopsy, the *H. pylori* status found in each type of sample was compared. A correlation was found between the *H. pylori* presence in biopsy and its presence in lavage fluid (Fisher’s exact test,  $p=0.015$ ), indicating lavage fluid is a valid alternative for determination of *H. pylori* infection.

The sensitivity and specificity of the lavage fluid analysis was calculated, considering biopsy as a golden standard. Fourteen patients, of which the lavage fluid as well as the biopsy was analyzed, were included into this calculation (patients 2, 4, 5, 6, 8, 10, 15, 23–25, 27–30, Table 1), which is shown in Table 4. Of the remaining patients, either the biopsy or the lavage fluid was missing;

therefore, these data cannot be used in the sensitivity/specificity calculation.

The sensitivity was 100%, which means that in case of the presence of *H. pylori* in the biopsy specimen, the lavage fluid analysis detected it in 100% of cases. The specificity of lavage fluid analysis was 66.7%, which means the chance for false-positives is over 30%. With regard to gender, age, BMI, history of PUD, location of perforation, complications after procedure, and use of steroids, PPI, or antihistaminic medication, no statistically significant correlation was found.

## Discussion

Concerning the role of *H. pylori* in the pathogenesis of PPU, some studies have been reported comparing the prevalence of *H. pylori* infection in patients with PPU to the prevalence in controls. They appear to be similar, suggesting that other factors like NSAID use play a role.<sup>19–21</sup> However, the substantial genetic heterogeneity

**Table 3** Distribution of the *vacA* s1 Subtypes

<i>VacA</i> Subtype	Fluid from Naso-Gastric Tube		Lavage Fluid		Gastric Tissue Biopsy		Control Non-Ulcer
	No.	%	No.	%	No.	%	%
S1a	8	80.0	13	92.9	5	71.4	81.3
S1b	2	20.0	1	7.1	2	28.6	18.7
S1c	0	0	0	0	0	0	0
Total	10	100	14	100	7	100	100

The s1a subtype is predominant in all sample types.

The “Control non ulcer” column represents the frequencies, found by van Doorn et al., in a population without PUD and is added to allow easy comparison.



**Table 4** Calculation of Sensitivity and Specificity of Lavage Fluid Analysis

		Biopsy		
		+	–	Total
Lavage fluid	+	8	2	10
	–	0	4	4
	Total	8	6	14
		Sens 8/8=1	Spec 4/6=0.67	

of *H. pylori* that has been revealed over the years leads to the hypothesis of a specific genotype causing PPU.<sup>5</sup> Controls might test positive for *H. pylori*, but not develop PPU because it would not be this specific genotype that is isolated. This study of a selected population of patients, all with PPU, shows a limited diversity of *H. pylori* genotypes as represented by Table 1.

*VacA* s1 strains are predominantly present in the three sample types of which s1a is the predominant subtype. Concerning the *vacA* m-region, the m1 strains are found in a majority of cases; however, the difference is less convincing than for *vacA* s1. Except for the biopsy samples, the *cagA* positive strains were predominantly present in this population. In the biopsy samples, the frequency of *cagA*-positive strains seemed to be low; however, this number is distorted because in two of nine positive biopsies, a decent comparison with the other samples was not possible. In patient 5, genotyping of the lavage fluid and nasogastric tube fluid was incomplete, and for patient 15, the opposite was the case. This means that the actual incidence should be 71.4 % (5/7).

Summarising, these results shows that the *vacA* s1, *cagA*-positive strains were predominant in this population of patients with PPU. This finding is in accordance with literature reporting correlations between the presence of *vacA* s1, *cagA*-positive strains and PUD.<sup>6,10</sup> Therefore, detection of the genotype *vacA* s1 does not specifically predict PPU; nevertheless, clinicians should be aware of this association.

In Tables 2 and 3 the genetic distribution in a Dutch population without PUD, as found by van Doorn et al., are added for comparison. The frequencies found in this study for *vacA* s1, m1, and *cagA*-positive strains are clearly higher than in the non-PUD group, confirming the aforementioned hypothesis. However, with regard to the subtypes, Table 3 shows an almost similar distribution of frequencies, suggesting that determination of the allelic subtype is of less importance.

In only 60% of patients biopsies could be analyzed. The reason for missing 40% is the restraint of the surgeon to take a biopsy when risk of bleeding and more difficult closure of the defect was estimated to be too high, which emphasizes the importance of finding an alternative. To do

so, the *H. pylori* status of the patient as determined by biopsy was compared to the status as determined by analysis of nasogastric tube fluid, lavage fluid, and serum. A statistically significant correlation was found between the *H. pylori* status in biopsy and its status in lavage fluid (Fisher's exact test,  $p=0.015$ ). This finding suggests that determination of the *H. pylori* status can be done with lavage fluid as well, obviously without any risk of bleeding and closure related difficulties. The sensitivity is 100%, but the specificity is 66.7%. This could mean the chance for false-positives is over 30%, which is not optimal and could lead to therapy overshoot. However, considering the fact that with the lavage a larger area is sampled, rendering the chance of positive test results higher than in biopsy, it is more likely to find false negative biopsy results. This could lead to a therapy undershoot, which obviates the importance having an alternative for a biopsy.

In only two samples, both nasogastric tube fluids, a *H. pylori* genotype was isolated, while  $\beta$ -globin tested negative. In nine samples (seven nasogastric tube fluid, two lavage fluid) both  $\beta$ -globin and *H. pylori* tested negative. This means that either no humane cells were present in the samples, which is unlikely, or that an error in the PCR procedure had occurred. Because this was unclear these results were considered unreliable. Therefore, it still could be possible that nasogastric tube fluid is a good alternative for determining the *H. pylori* status as well.

Overall, these results are positive, however they should be confirmed in a larger population.

## Conclusion

This study shows that in a population of 30 patients with PPU, *vacA* s1, *cagA* positive strains are predominant. This finding is in accordance with literature reporting correlations between the presence of *vacA* s1, *cagA*-positive strains, and PUD. Therefore, detection of this genotype does not specifically predict PPU. Nevertheless, clinicians should be aware of this association.

This study shows as well that it is feasible to use intraoperative lavage fluid to determine the *H. pylori* status of the patient, implicating that biopsies, with a risk of bleeding and more difficult closure of the defect, are not necessary anymore. In addition, considering the fact that a larger area is sampled with lavaging, biopsies may result in more false negative results leading to insufficient therapy.

**Acknowledgments** The authors would like to thank W.A. Bemelman (Department of Surgery, University of Amsterdam, Amsterdam, The Netherlands), A. van der Ham (Department of Surgery, Sint Franciscus Gasthuis, Rotterdam, The Netherlands), I. Oei (Department of Surgery, Reinier de Graaf Gasthuis, Delft, The Netherlands), and Bianca Gieze (Crucell, Leiden, The Netherlands) for their input in this study.

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