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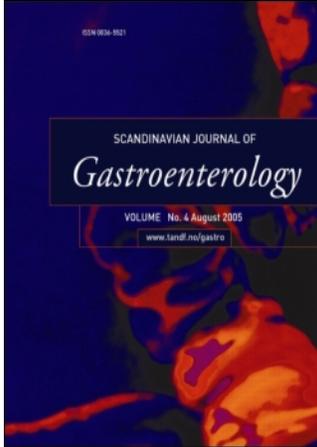
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ORIGINAL ARTICLE

Persistence of *Helicobacter pylori* infection in patients with peptic ulcer perforationHELENA ANDRESON¹, TOOMAS SILLAKIVI², MARGOT PEETSALU²,
ANTS PEETSALU² & MARIKA MIKELSAAR¹¹Department of Microbiology, and ²Department of Surgery, University of Tartu, Tartu, Estonia**Abstract**

Objective. In patients with perforated peptic ulcer (PPU) the convergence between the high eradication rate of *Helicobacter pylori* infection and low rates of ulcer relapse after treatment has been associated with reinfection by non-virulent strains. The objective of this study was to evaluate the persistence of infection by virulent *H. pylori* strains and ulcer recurrence in 33 patients with PPU one year after surgery and antimicrobial treatment. **Material and methods.** The histological evaluation and molecular detection of *H. pylori* *cagA* and *ureA* genes, *vacA* allelic types and the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analyses of the *glmM* gene products from antral mucosa specimens were performed initially, 2–5 months and 1 year after therapy. **Results.** The density of *H. pylori* colonization was temporarily decreased ($p < 0.05$) 2–5 months after therapy. After one year, complete eradication was achieved in only 7 patients (23%) at histological examination and recurrent ulcers were found in 3/33 (9%) patients. The *vacA* s1a allelic type of *cagA*-positive strains persisted in 19/33 (58%) PPU patients with identical PCR-RFLP fingerprints in 8/9 (89%) of the patients. **Conclusions.** In PPU patients with a low eradication rate of *H. pylori* infection after surgical and antimicrobial treatment, the frequent recrudescence of the infection is mostly caused by the persisting virulent strains of the *cagA* and *vacA* s1a subtypes. In the 1-year follow-up period the recurrent ulceration can be postponed just by the lowered colonization density of *H. pylori* after eradication therapy.

Key Words: *cagA*, *Helicobacter pylori*, perforated peptic ulcer, persistent infection, *vacA*, virulence

Introduction

Helicobacter pylori infection is closely associated with chronic gastritis, non-complicated peptic ulcer disease (PUD) and gastric cancer. Similarly, in the case of perforated peptic ulcer (PPU), which is a potentially fatal complication of PUD (both duodenal and gastric ulcer), the prevalence of *H. pylori* is high, varying between 50% and 96% [1–4]. Using classical quadruple therapy, a high *H. pylori* eradication rate, 80–98%, has been reached 8 weeks after treatment. One year later, however, the data on outcome, e.g. ulcer relapse rate, are quite different: 33% [5] versus 5% [6].

The contradictory results reported on the incidence of recurrent ulcer after surgical intervention and eradication treatment in PPU patients [3,5–7] may be attributed to different infection rates in

populations where the high prevalence could facilitate the reinfection. Moreover, the precise clinical relevance of persistent infections or reinfections is unknown. Reappearance of the microbe without associated ulcer recurrence has been explained by reinfection with some non-virulent strains [8,9].

In PUD, *H. pylori* strains possess the cytotoxin-associated gene (*cagA*) with the vacuolating cytotoxin gene (*vacA*) of the s1/m1 allelic combination [10], while the *vacA* type s1a strains appear to be more cytotoxic than the s1b or s2 strains [11]. In our previous study, no significant differences were found in the *cagA* and *vacA* markers between the strains from the perforated and non-complicated peptic ulcer groups [12]. There are no such investigations where molecular virulence markers were applied for differentiation between true reinfection with a new strain and recrudescence when eradication of the

permanent strain of *H. pylori* in PPU patients failed. Populations with a high *H. pylori* incidence and hence at risk for reinfection, like Estonia [13], provide a good opportunity for elucidation of the problem in the course of follow-up studies after treatment.

In our study the aim was to evaluate the putative eradication, persistent infection or reinfection by *H. pylori* and ulcer recurrence in patients with PPU in a 1-year follow-up study after surgery and antimicrobial treatment. The polymerase chain reaction (PCR)-based molecular virulence markers, the restriction fragment length polymorphism (RFLP) pattern and the colonization density of *H. pylori* were established directly from gastric antrum mucosa samples.

Material and methods

Patients

The study comprised 33 persons (29 M, 4 F, mean age 43 ± 14) retrospectively selected from among the patients operated on for PPU and observed clinically during the period 1997–2001 in the Clinic of Surgery of Tartu University Hospital. The inclusion criteria were agreement to participate in the study and availability of three consecutive gastric mucosa biopsy samples during the 1-year period (initial, 2–5 months and 1 year later), the first probe being *H. pylori* positive as detected by histology and PCR.

The perforated ulcers were classified as gastric (2 cases) and duodenal ulcers (31 cases) according to the location. The surgical therapy of 18 patients included definitive (truncal vagotomy combined with drainage procedure) and in 15 patients non-definitive (ulcer excision or suturation) operations. In the latter case, traditional triple therapy (amoxicillin 1000 mg b.i.d., metronidazole 500 mg q.i.d. or clarithromycin 500 mg b.i.d. and omeprazole 20 mg b.i.d.) was applied for 7 days. In vagotomized patients different antimicrobial treatment schemes without omeprazole were used for 5 to 7 days [14]. Eradication was defined as a negative histological finding of *H. pylori* colonization during the 1-year follow-up.

Material

Two gastric biopsy samples of the antral mucosa were taken intra-operatively through the perforation, or postoperatively on panendoscopy, before eradication treatment, for histological evaluation and molecular analysis. Similarly, the second and the third biopsy samples were taken on endoscopy 2–5 months and 1 year after the first intervention.

The biopsy samples for molecular analysis were placed into a lysis buffer (200 mM Tris-HCl (pH 8.0), 25 mM EDTA, 300 mM NaCl, 1.2% sodium dodecyl sulphate) and stored at -20°C .

Histological evaluation

Ninety-five samples from the antrum were used for histological evaluation. Owing to technical failure, four histology preparations from three of the patients (1 from initial, 3 from 2–5 month samples) were unavailable. The biopsy specimens were fixed overnight in neutral buffered formalin, dehydrated and embedded in paraffin. Tissue sections were stained using the modified Giemsa method for further semiquantitative assessment of *H. pylori* colonization as described earlier [15]. The colonization of the antral mucosa by *H. pylori* was graded as follows: grade 0, absence of *H. pylori*; grade 1 (low or mild), <20 microbes per field; grade 2 (moderate), 20–60 microbes per field; grade 3 (high or severe), >60 microbes per field.

Extraction of DNA

Ninety-nine antral mucosa biopsy samples were collected for molecular analysis. DNA was extracted from the frozen gastric biopsy specimens using the procedure described in a previous study [16].

Primers

The presence of *cagA* in each strain was determined by PCR using D008 and R008 primers [17]. The *vacA* signal- and midregion were typed by PCR [10]. The primers VA1-F, VA1-R and SS2-F were used to categorize the *vacA* s region as type s1 or s2. For subclassification of the s1 allele, the reverse primer VA1-R was used together with the allelic subtype-specific forward primers (SS1-F primer for the subtype s1a and SS3-F primer for the subtype s1b). The *vacA* midregion was typed by PCR as m1 (primers VA3-F and VA3-R) or m2 (primers VA4-F and VA4-R). The presence of *H. pylori* was confirmed by the primers HPU1 and HPU2 for detecting the urease A gene (*ureA*) [18]. For RFLP, the 1,169-bp fragment of the *glmM* (formerly *ureC*) gene was amplified with the primers ureC-U (5'-AAG AAG TCA AAA ACG CCC CAA AAC-3') and ureC-L (5'-CTT ATC CCC ATG CAC GAT ATT CCC-3') [19].

PCR conditions and amplification

The reaction mixtures were prepared and the PCR reactions were performed for the *ureA*, *cagA*, s1a and s1b primer pairs [16]. Thermal cycling for the

vacA primer pairs (except s1a and s1b) comprised 4 min of preincubation at 94°C, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min 30 s, and a final extension of 72°C for 4 min. For amplification of the 1,169-bp fragment of the *glmM* gene, initial preincubation at 94°C for 5 min was followed by 35 cycles of 94°C for 45 s, 59°C for 30 s, 72°C for 1 min 30 s, and final extension of 72°C for 10 min. The PCR products were identified by electrophoresis on 1.5% agarose gels in the case of the 1.169-bp PCR fragment and 2% agarose gels were used for all other PCR products.

PCR-RFLP analysis

Initial and follow-up samples with a sufficient concentration of DNA were selected from 10 patients for further PCR-RFLP analyses [19]. The PCR-amplified 1,169-bp products of the *glmM* gene were precipitated in ethanol, the pellets were washed with 70% ethanol, and dissolved in 5 µl water. The purified PCR products obtained were digested with the restriction enzyme HhaI (Fermentas AB, Lithuania) with the recommended Tango buffer at 37°C for 20 h. The digested DNA fragments were analysed using electrophoresis on 3.5% agarose gels (Agarose MP; Roche Diagnostics, Germany).

Statistical analysis

Differences in the treatment schemes and the association between the *cagA* and *vacA* genes were analysed using the Fisher exact test. The χ^2 test was used for comparison of the PCR and histology methods. The data on *H. pylori* colonization density for different treatment groups were analysed with the Mann-Whitney rank sum test. Significance was set at a *p*-value of less than 0.05. The Statistica 6.0 (StatSoft Inc., USA) software program was used for all analyses.

Ethics

The study was approved by the Tartu University Research Ethics Committee and informed consent was obtained from each participant in all cases.

Results

Clinical and histological data

The follow-up of *H. pylori* eradication showed that in 21 patients out of 30 (70%) 2–5 months after treatment and in 23 patients (77%) one year after treatment *H. pylori* was still present in mucosal samples (3 patients with unavailable histology data were excluded from this analysis). The patients in

the triple therapy group showed slightly better results of *H. pylori* eradication (6/15, 40% versus 1/15, 7%). At 2–5 months after therapy the colonization density was temporarily reduced ($p < 0.05$) in both treatment groups, but by the time of the final evaluation colonization had increased in the group that had received modified treatment ($p < 0.05$) (Figure 1).

Recurrent duodenal ulcer was found in only 3 patients out of 33 (9%) in both treatment groups. In two cases with non-eradicated *H. pylori*, recurrent ulcer was detected after one year and in one patient at both follow-up endoscopic examinations after surgical and eradication triple therapy.

H. pylori ureA, vacA and cagA markers

Judging by the detection of *ureA* and *vacA* genes, all samples were initially *H. pylori* positive. *H. pylori* positivity was found in 19 (58%) patients out of 33 at the 2–5-month follow-up and in 23 (70%) patients at one year. None of these findings differed ($p > 0.05$) from those of the histological evaluation.

Initially, the majority of patients (31/33) were infected with *cagA* positive strains of the *vacA* s1a allelic type. All signal- and midregion *vacA* alleles were found, with the exception of s1b. The dynamics of *H. pylori* infection by PCR and the distribution of the *vacA* subtypes, comprising five different allelic combinations are summarized in Table I. At the first and at the last follow-up, infection with the same *vacA* virulence markers persisted in most of the patients, 19/33 (58%). In only 4/33 (12%) patients,

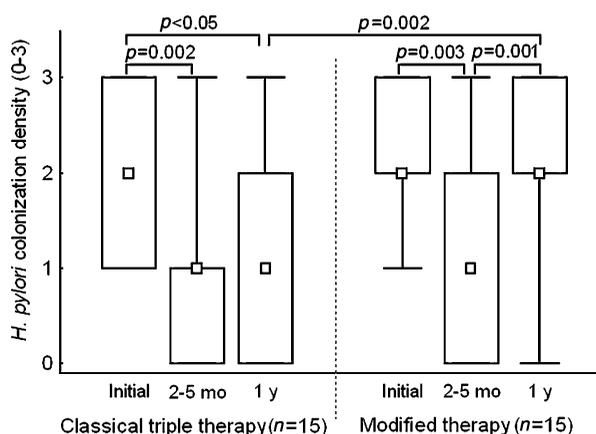


Figure 1. *H. pylori* colonization density in patients with perforated peptic ulcer (PPU) during 1-year follow-up in the case of classical and modified therapies. Initial biopsy samples before therapy, 2–5 months (mo), and one year (y) after therapy. *H. pylori* density grades are shown as boxes: internal points, medians; tops and bottoms of boxes, 75th and 25th percentiles, respectively; upper and lower bars, 90th and 10th percentiles, respectively.

Table I. Dynamics of *H. pylori* infection and strains in the follow-up samples of the patients with perforated peptic ulcer (PPU).

Dynamics of the infection	<i>n</i> = 33*	<i>H. pylori vacA</i> subtypes in follow-up samples		
		1 (operation)	2 (2–5 months)	3 (1 year)
Eradication of <i>H. pylori</i> (<i>n</i> = 10)	1	s1a/m1	s1a/m1	Neg.
	4	s1a/m1	Neg.	Neg.
	4	s1a/m2	Neg.	Neg.
	1	s2/m2	Neg.	Neg.
	1	s1a/m1	s1a/m1	s1a/m1
Persistent <i>H. pylori</i> (<i>n</i> = 19)	8 ⁵	s1a/m1	s1a/m1	s1a/m1
	3 ²	s1a/m2	s1a/m2	s1a/m2
	1 ¹	s2/m2	s2/m2	s2/m2
	1	s1a	s1a	s1a
	2	s1a/m1/m2	s1a/m1/m2	s1a/m1/m2
	4 ¹	s1a/m1	Neg.	s1a/m1
Reinfection (<i>n</i> = 4)	1	s1a/m1	s1a/m1	s1a/m2
	2 ¹	s1a/m1	s1a/m1	s1a
	1	s1a/m1	Neg.	s1a/m2

*The superscript indicates the number of patients enrolled for further PCR-RFLP analysis.

possible reinfection with the *H. pylori* virulence markers other than the initial ones was detected.

PCR-RFLP typing

We fingerprinted the initial and the follow-up samples from 10 patients: 9 belonged to the group of persistent *H. pylori* with similar virulence markers and one with different ones. The latter patient, initially with the *H. pylori* s1a/m1 strain, showed different patterns both by PCR (s1a allele without the m allele) and PCR-RFLP (Table I; Figure 2, patient 5). Among the 9 patients with similar virulence markers, the overwhelming majority (8/9) displayed no differences in the restriction pattern between the initial biopsy sample and the follow-up sample (Figure 2, patients 1, 3, 4 and 6–10). The PCR-RFLP patterns of the follow-up samples were different from the initial ones in only one case, and this was due to the variance of one band (Figure 2, patient 2).

Discussion

The purpose of our study was to evaluate the change or persistence of *H. pylori* virulent strains in patients with PPU one year after surgery and antimicrobial treatment and the recurrence of peptic ulcer. Direct gastric mucosa samples were investigated by histological and molecular methods. We found that in our patients one year after treatment the eradication of *H. pylori* in those with PPU was rarely (23%) achieved and the same strains continued to persist. This result is quite different from the above-mentioned high eradication rate (84%) registered at 2-month follow-up [6].

It would be beyond the aim of our study to discuss the success rate of different *H. pylori* treatment methods, as traditional means and duration of treatment of PPU patients were applied. Yet, we registered that the density of *H. pylori* was significantly lowered 2–5 months after therapy even when different antimicrobial treatment schemes were applied. Therefore, it could be speculated that,

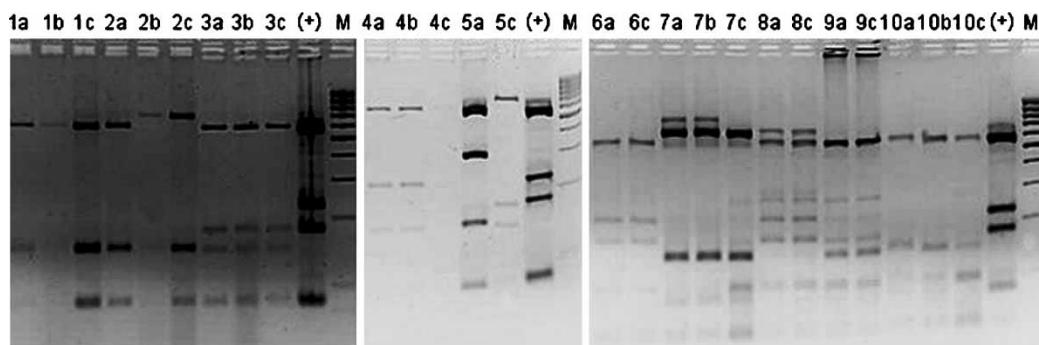


Figure 2. Restriction fragment length polymorphism (RFLP) patterns of the follow-up samples from 10 patients with perforated peptic ulcer (PPU). (a) Before therapy, (b) 2–5 months after therapy and (c) 1-year follow-up; the patients are indicated by numerals. PCR-amplified 1,169 bp fragments of the *glmM* gene of *H. pylori* were digested with the *HhaI* restriction enzyme. Lane M is the 100 bp DNA ladder (Fermentas AB) and lane (+) is the *H. pylori* strain NCTC 11637 as the positive control.

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contrary to previous suggestions [8], the time-span of 4 to 8 weeks is not sufficient to confirm eradication. In our study, the density of colonization started to return to the values of the original samples after the 1-year period from both treatment groups but more so in the group that received modified therapy. At the same time, these results indicate that the lowered susceptibility of *H. pylori* strains could not be the reason for treatment failure, which remains to be elucidated.

In addition to a histological evaluation, we explored the direct samples of the gastric mucosa for molecular detection of persistent colonization by *H. pylori*. The use of the PCR method supported the data of histological evaluation, showing that in most (58%) of the PPU patients *H. pylori* infection persisted for one year after surgery and eradication therapy. Previously, Björkholm et al. [20] described the ability of *H. pylori* of increased virulence to survive inside epithelial cells. This could also be the case with the PPU patients studied by us in whom mostly the highly virulent *cagA* positive strains (94%) with the *vacA* s1a/m1 alleles prevailed (68%). It is possible that, intracellularly, the bacterium can partly evade antimicrobial therapy, at the same time showing decreased colonization in follow-up diagnostics shortly after therapy. Recently, this possibility was also suggested by Gisbert [8].

In our PPU patients, who displayed a fairly low eradication rate of *H. pylori* infection, the rate of ulcer relapse (9%) was still not significantly different from the corresponding data (5%) of a study where a markedly higher eradication rate was reported [6]. The rate of possible reinfection according to molecular analysis turned out to be low (12%), showing that in communities with a high prevalence of *H. pylori* but yet good standards of hygiene, development of the infection usually occurs with one strain. Hence our study, which revealed a low rate of ulcer relapse, did not confirm the assumption [8,9] that new non-ulcerogenic strains (*vacA* s2 alleles) can colonize the gastric mucosa after therapy. Apparently, the lowered density after eradication therapy postpones development of recurrent ulceration in the 1-year follow-up period irrespective of recrudescence of presence of ulcerogenic strains. Moreover, our study showed a mixed colonization pattern in only 6% of the patients (Table I). These results are in good accordance with those of studies comparing the pre- and post-treatment strains of *H. pylori*, where approximately 80% of the strains were shown to be identical [8].

During follow-up we found that in two patients the usual *vacA* s1a/m1 allelic combination in the initial PCR sample was replaced by a strain of the mere s1a subtype. PCR-RFLP analysis confirmed

this difference in one tested patient, yielding clearly distinguishable RFLP patterns for the two samples. Similarly, one patient had persistent infection with the s1a subtype of *H. pylori* in all three follow-up samples without the mid-region allele of the *vacA* gene. It is possible that with the primers used we could have missed some midregion variants described previously in addition to the m1 and m2 alleles [21–23].

Application of direct samples of gastric biopsy for detection of the RFLP pattern has been shown to be effective for differentiating between reinfection and the persistent strains of *H. pylori* after using different therapy schemes in PUD patients or in patients with upper gastrointestinal complaints [19,24,25]. In the present study we compared the restriction pattern of the *glmM* gene in nine patients with persistent *vacA* alleles before and after therapy. In most patients (89%) the RFLP profiles of the subsequent samples were indistinguishable. We found only one patient for whom there was a minor one-band difference between the initial sample and the two follow-up samples, despite the fact that they were of the same *vacA* genotype.

Conclusions

In PPU patients with a low eradication rate of *H. pylori* after surgical and antimicrobial treatment, the frequent recrudescence of the infection is mostly caused by the persisting virulent strains of the *cagA* and *vacA* s1a subtypes. In the 1-year follow-up period the recurrent ulceration may be postponed just by lowered colonization density of *H. pylori* after eradication therapy.

Acknowledgements

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