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Diversity of *Helicobacter pylori* genotypes among Estonian and Russian patients with perforated peptic ulcer, living in Southern Estonia

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Abstract

To compare the genomic variation of *Helicobacter pylori* in samples obtained from patients with perforated peptic ulcer, living in the same area of Estonia but belonging to different nationalities, 50 non-consecutive patients (32 Estonians and 18 Russians) admitted in the Tartu University Hospital in 1997–1999 were studied. Gastric samples of antral mucosa were obtained during operation and analysed histologically and with PCR for detection of different genotypes of *H. pylori* (*cagA* and *vacA* s and m subtypes). Among the 50 perforated peptic ulcer patients with histologically proven *H. pylori* colonisation no sample of gastric mucosa showed the s1b subtype of the *vacA* gene. The perforated peptic ulcer patients were mainly infected with *cagA* (82%) and s1 (98%) genotypes of *H. pylori*. The distribution of s1a/m1, s1a/m2 and s2/m2 subtypes of *vacA* genes was statistically different in Estonian and Russian patients ($P < 0.05$). In conclusion differences in the distribution of *vacA* s and m subtypes of *H. pylori* were revealed between Estonian and Russian patients with perforated peptic ulcer from Southern Estonia.

Keywords: PCR; Ethnic tropism; *cagA*; *vacA*; *Helicobacter pylori*

1. Introduction

Helicobacter pylori plays an important role in gastritis, peptic ulcer disease and gastric malignancy [1]. To date, several products of *H. pylori* genes (*vacA* and its subtypes, *cagA*, *ice*), acting as disease-associated pathogenic factors in infected persons, have been identified [2–4]. A key feature of *H. pylori* is the enormous genomic micro-diversity of its strains distributed over the globe, even many individuals appear to be infected by a unique strain.

At the same time, different countries seem to be characterised by a particular geographic pattern of different *H. pylori* genotypes [5,6]. In Western populations, gastric atrophy, duodenal ulceration, intestinal metaplasia, gastric carcinoma are more common among patients infected with *cagA* positive than among patients infected with *cagA* negative strains [4,5]. Yet, in China and Japan the association between *cagA* positivity and virulence of *H. pylori* strains was equally frequent among both diseased and control patients [5,7]. Recently, it was shown that in East Asia and in the Western countries, distinct variants of *H. pylori cagA* genes were associated with particular *vacA* subtypes [8].

However, the clinical significance of the genetic markers of *H. pylori* has not yet definitely been proved. Particularly, the incidence of peptic ulcer disease is not reflected in the frequency of different *H. pylori* lineages among Polynesians and European New Zealanders [9]. Obviously, further research is needed to compare differences in the *H. pylori* genome between particular ethnic groups with the same underlying disease, living in the same geographic region.

In Estonia, an increase in perforated peptic ulcer (PPU) has been noted since 1991–1997, the mean incidence for Southern Estonia amounting to 21/100 000 [10]. At the same time, in Western countries the incidence of PPU

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has been kept in the range less than 10/100 000 in recent decades [11]. Estonia is a state in Eastern Europe, lying on the coast of the Gulf Finland, with a population of about 1.5 million and area of 45 000 km^2. The Estonian population consists of two-thirds of native Estonians and one-third of other nationalities, mainly Russians. In the present study an attempt was made to compare the genomic variation of *H. pylori* in samples obtained from patients with PPU, living in the same area of Estonia but belonging to different nationalities.

2. Materials and methods

2.1. Patients

The study was carried out at Tartu (Southern Estonia) University Hospital from January 1, 1997 to December 31, 1999. During this period 129 patients with PPU were hospitalised. Informed consent was obtained from all patients who were questioned using a standard questionnaire. Only the patients ($n = 53$) of whom both parents could be identified as being of the same nationality were included. All patients were operated and three gastric specimens of the antral mucosa were taken intra-operatively through the perforation, or postoperatively on pan-endoscopy. Two of the specimens were analysed by histological methods and one by molecular methods.

2.2. Histological evaluation

Biopsy specimens were fixed overnight in neutral buffered formalin and embedded in paraffin. Tissue sections were stained by the modified Giemsa method for semi-quantitative assessment of *H. pylori* colonisation as described earlier [12].

2.3. Molecular evaluation

For analysis with molecular methods the specimens were placed into 500 μl of lysis buffer (200 mM Tris–HCl (pH 8.0), 25 mM EDTA, 300 mM NaCl, 1.2% sodium dodecyl sulfate) and stored at −20°C. Different genotypes (*cagA* and *vacA* s and m subtypes) of *H. pylori* were assessed. DNA was extracted from a frozen gastric biopsy specimen using the following procedure: 10 μl of the proteinase K (400 μg ml^{-1}) was added to the biopsy specimen suspended in the lysis buffer (500 μl). The mixture was incubated at 37°C for 24 h. The lysate was extracted with an equal volume of phenol–chloroform and precipitated with ethanol. A DNA pellet was collected by centrifugation, washed with 70% ethanol and finally resuspended in 35–50 μl of TE buffer (10 mM Tris–HCl (pH 8.0), 0.1 mM EDTA). For analysis of the s and m regions of *vacA* and for detection of the *cagA* gene, the primers shown in Table 1 were used.

2.4. PCR conditions and amplification

Reaction mixtures were prepared in a volume of 50 μl, containing 0.3 μM concentration of each primer; 0.2 mM concentration of deoxynucleoside triphosphates; 10× reaction buffer (200 mM Tris–HCl (pH 8.5), 500 mM KCl, 10 mM $MgCl_2$, 0.01% (w/v) gelatin); 1 mg ml^{-1} albumin (BSA); 2.5 mM $MgCl_2$; 5 U of Taq DNA polymerase (Fermentas); and ∼10 ng DNA sample. The mixtures were placed in a PCR thermocycler (Biometra, Eppendorf). Thermal cycling for all primer pairs comprised 4 min of preincubation at 95°C, followed by one cycle of 1 min at 95°C, 1 min 10 s at 56°C, 30 s at 72°C; three cycles of 1 min at 95°C, 1 min 10 s at 54°C, 30 s at 72°C; 36 cycles of 1 min 95°C, 1 min 10 s at 52°C, 30 s at 72°C. PCR products were identified by electrophoresis on 2% agarose gels.

2.5. Statistical analysis

The mean values of patients' age were compared by employing the Student *t*-test. The absolute figures of prevalence were compared by using the Chi-square test with a continuity correction. Differences were considered statistically significant for *P* values less than 0.05.

Table 1
PCR primers for amplification of *vacA* sequences and *cagA* gene of *H. pylori*

Amplified region	Primer destination	Primer sequence	Product size (bp)	Source of reference
s1	VA1-F	ATGGAAATACAACAAACACAC		[2]
	VA1-R	CTGCTTGAATGCGCCAAAC	259	
s1a	SS1-F[a]	GTCAGCATCACACCGCAAC	190	
s1b	SS3-F[a]	AGCGCCATACCGCAAGAG	187	
s2	SS2-F[a]	GCTAACACGCCAAATGATCC	199	
m1	VA3-F	GGTCAAAATGCGGTCATGG		
	VA3-R	CCATTGGTACCTGTAGAAAC	290	
m2	VA4-F	GGAGCCCCAGGAAACATTG		
	VA4-R	CATAACTAGCGCCTTGCAC	352	
cagA	D008	ATAATGCTAAATTAGACAACTTGAGCGA		[13,14]
	R008	TTAGAATAATCAACAAACATCACGCCAT	297	

[a]Used in combination with primer VA1-R.

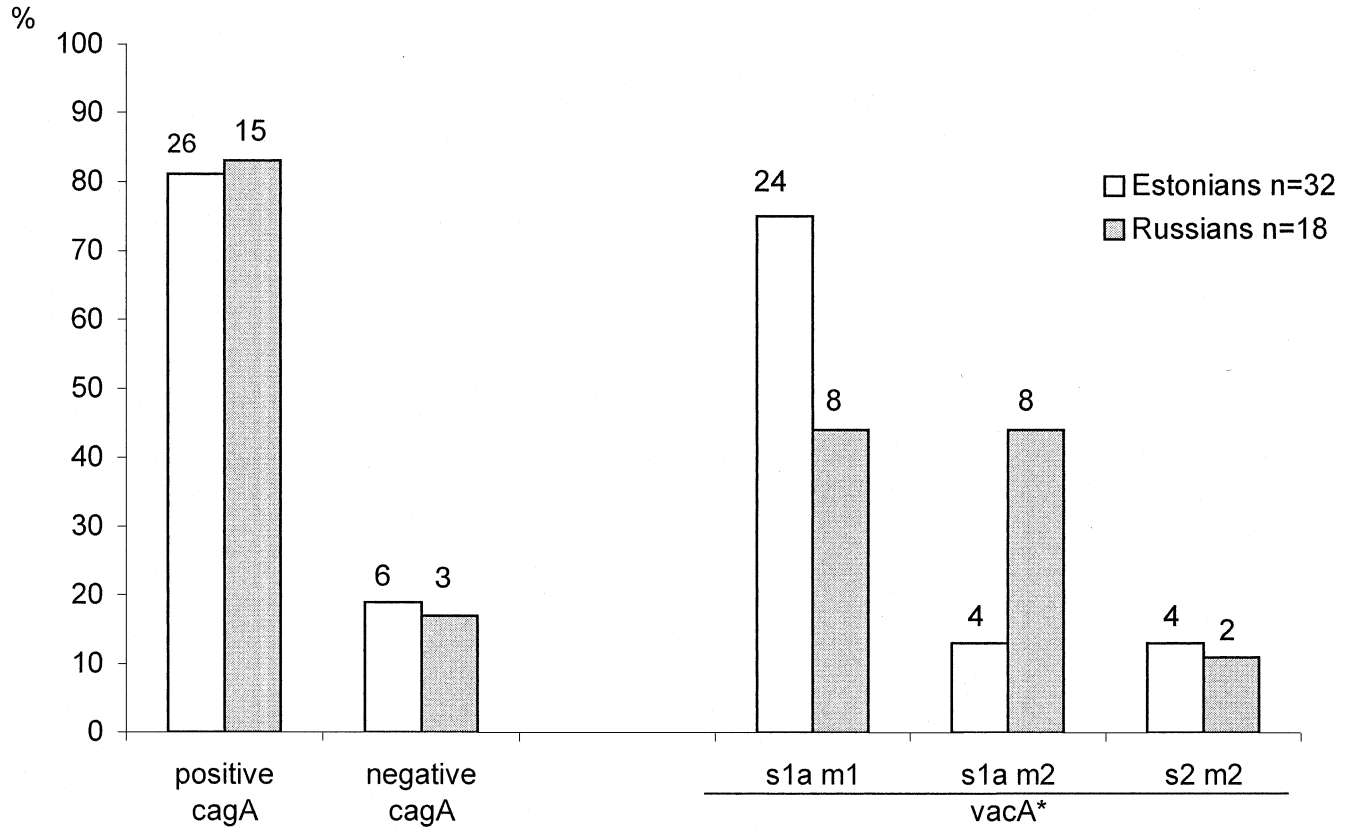

Fig. 1. *cagA* and *vacA* status of *H. pylori* in antral mucosa samples of Estonian and Russian patients with PPU (**vacA* subtypes distribution difference $P = 0.037$).

3. Results

H. pylori was detected histologically in the antral mucosa of 52 out of 53 included PPU patients (98%). The degree of colonisation in the antral mucosa varied from grade 1 to grade 3 (grade 1: <20 microbes per field; grade 2: 20–60 microbes per field; grade 3: >60 microbes per field).

Using a PCR technique *H. pylori* was detected in the antral mucosa of 51 PPU patients (96%). In one patient with no detected *vacA* or *cagA* genotype, *H. pylori* was still proven to be present histologically.

In one Estonian male with a *cagA* positive sample, two *vacA* gene subtypes (s1a/m1 and s1a/m2) were found simultaneously and the patient was excluded from further analysis when comparing *H. pylori* subtypes in persons of different nations. The distribution of the remaining 50 *H. pylori* positive samples from 32 Estonians and 18 Russians according to the markers of *cagA* and *vacA* gene subtypes is presented in Table 2. Among *H. pylori* positive samples, 41 (82%) were *cagA* positive. The most frequent was the s1a/m1 *vacA* subtype (32 cases), while the s1b subtype was not found in our PPU patients.

Both s1 subtypes (s1a/m1 and s1a/m2) prevailed (98%) in *cagA* positive samples. Comparison of the distribution of the pattern of three *vacA* subtypes in patients with *cagA* positive and negative strains by using Chi-square test yielded statistical difference at the level $P < 0.001$ (Table 2).

No differences were observed in the distribution of *cagA* positive or negative markers between Estonian and Russian patients with PPU, as the *cagA* gene was revealed in 81% (26 of 32) and 83% (15 of 18) of cases, respectively (Fig. 1). In contrast, the distribution of *vacA* subtypes was different in the gastric samples of our Estonian and Russian patients. The s1a/m1 subtype was found in 75% (24 of 32) of Estonians but in only 44% (8 of 18) of Russians. At the same time, the s1a/m2 subtype was more frequent in

Table 2
Distribution of *H. pylori cagA* and *vacA* status in PPU patients

vacA subtype	$cagA^+$, $n = 41$	$cagA^-$, $n = 9$	Chi-square
s1a/m1	29 (71%)	3 (33%)	
s1a/m2	11 (27%)	1 (11%)	$P < 0.001$
s2/m2	1 (2%)	5 (56%)	
Total	41 (100%)	9 (100%)	

Russians (44%, eight cases) than in Estonians (13%, four cases). The s2/m2 subtype was detected in four (13%) Estonians and two (11%) Russians. Comparison of the distribution of three *vacA* subtypes in the gastric mucosa samples of PPU patients of different nationalities using Chi-square test yielded statistical difference ($P = 0.037$). No significant differences were revealed in the distribution of age, sex, smoking habits or ulcer location among our Estonian and Russian PPU patients.

4. Discussion

We investigated the distribution of *H. pylori* genotypes in Southern Estonia among patients with complicated, histologically proven *H. pylori* associated peptic ulcer disease. Using PCR method the genotypes of *H. pylori* were estimated according to the presence of the *cagA* gene and *vacA* gene subtypes in specimens of gastric antral mucosa.

The fact that we did not find any gastric mucosa sample containing the s1b subtype of the *vacA* gene among the 50 PPU patients investigated by us confirms the existence of geographical differences between *H. pylori* genotypes. Also, although the s1b subtype was not found in *H. pylori* from Asian patients [15], it has been established in several studies conducted in Portugal, The Netherlands, and the USA [3,9,16]. However, as Estonia is situated in Eastern Europe, geographic differences between *H. pylori* genotypes cannot be linked with different continents.

Furthermore, in our study the distribution of s1a/m1, s1a/m2 and s2/m2 subtypes of *vacA* genes was statistically different in Estonian and Russian patients ($P < 0.05$), both groups living in South Estonia. This confirms data about the ethnic tropism of *H. pylori*, suggested by Campbell et al. [9]. Yet these authors associated differences between the *H. pylori* strains colonising Polynesians and Europeans in New Zealand, with race-specific specialisation of *H. pylori* separate strains. However, this cannot be the reason for *H. pylori* related ethnic differences between Estonians and Russians, as far as both nationalities belong to the same race. We suppose that different predominant strains may be circulating in a particular area.

The Estonian and Russian PPU patients studied by us showed similarity in the distribution of age, sex and smoking habits as well as ulcer localisation. Evidently, these factors are not closely associated with ethnic tropism of *H. pylori*. Regrettably, we could not follow the socio-economic conditions in case of our patients and hence it is impossible to conclude if their habits (difference in food or health status etc.) have influenced the distribution of *H. pylori* subtypes. In previous epidemiological studies these conditions were strongly associated with transmission of *H. pylori* infection. In Germany, *H. pylori* infection was established in 6.1% of native German children versus 44.8% of Turkish children [17]. However, the different incidence of *H. pylori* infection in populations from the same country but having a different economic background does not explain spread of genetically distinct *H. pylori* strains among these populations. Whether the other determinants, e.g. region/country of birth and childhood, or microbial adherence and host receptors, determine a particular distribution of different genotypes of *H. pylori* should be established in further studies with a larger number of subjects.

The virulence markers differentiating *H. pylori* strains in patients with complicated and uncomplicated peptic ulcer have not been assessed up to now. In our study the *cagA* gene of the *H. pylori* genome was found in 82% of PPU patients. The *cagA* status was similar in samples from Estonian and Russian patients with the same underlying disease, while the *vacA* s1 subtype prevailed in both nationalities (98%). High prevalence of the $cagA^+$ genotype and *vacA* s1 subtype has been associated with increased virulence of *H. pylori* strains [2,3]. However, recently we found predominance of the *vacA* s1 gene in another sample of South Estonian patients suffering either from chronic active gastritis or peptic ulcer disease [18]. It is likely that the presence of *cagA* and *vacA* s1 genes of *H. pylori* strains is not exclusively specific for complicated peptic ulcer disease but may merely reflect the circulation of predominant strains in a particular geographic region of Estonia.

In conclusion, we found diversity in *H. pylori* genotypes among Estonian and Russian patients with PPU from South Estonia. This finding points to the need of further investigations for future development of novel therapeutic targets and vaccines specific for different ethnic groups.

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