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The evaluation of *vacA* gene alleles frequency in *Helicobacter pylori* strains in children and adults in Podlaskie region

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Abstract: The frequency of *Helicobacter pylori* infection in population can depend on the organism resistance, genetic condition, and bacterial strains virulence. A *vacA* gene, of mosaic structure, which encodes vacuolating cytotoxin is one of the known genes of *H. pylori*. The existence of several different genotypes of s and m regions enables the formation of numerous combinations of *vacA* gene genome. The studies on *vacA* genotype revealed that the frequency of occurrence of *H. pylori* containing *s1* or *s2*, as well as *m1* and *m2* alleles varies in different parts of the world. The aim of the studies performed in the group of children and adults was to evaluate the prevalence of particular *vacA* gene alleles distribution in the population of the Podlasie province. The allele *s1*, which occurred in 84.3% of the examined group (86.8% in children and 81.3% in adults), turned out to be the most frequently observed of the signal encoding region. Statistically significant differences in *s1* and *s2* alleles distribution in relation to a dwelling place were not detected. The allele *m2* (42.1% in children and 59% in adults) was the allele of midregion, most frequently occurring in our studies. The allele *m2* was observed more often in *H. pylori* strains in the inhabitants from the urban areas (data statistically significant).

Key words: Allele - *vacA* - *Helicobacter pylori*

Introduction

Helicobacter pylori infection of the gastroduodenal tract occurs in humans all over the world and concerns all social groups [1]. However, clinical manifestations and morphological features of gastric or duodenal pathology have been found to correlate with different virulence of the microorganisms. Bacterial virulence, strongly dependent on genes structure and expression of the relevant proteins, relates to different extent of *H. pylori* infection and processes involving gastroduodenal pathology.

There are two proteins of *H. pylori* recognized as most virulent that are crucial in the formation of lesions of gastric mucosa: vacA (vacuolating cytotoxin vacA) and CagA (cytotoxin-associated geneA). Both of them take part in the colonization and modulation of inflammatory response and in the development of inflammatory changes, peptic ulcer and gastric carcinoma [2].

The gene, encoding vacA protein, is present in the genome of all *H. pylori* strains, although some of strains do not induce the vacuolization effect in eukaryotic cells. In such cases transcription of *vacA* gene takes place on a considerably lower level than in phenotypic Tox+ strains. It is suspected that *vacA* gene activator can exist in (Tox+) strains, whereas a represor - in Tox- [2].

A gene, which encodes a vacuolating cytotoxin, contains areas of conservative and variable nucleotide sequences characteristic for mosaic structure [3]. In all *H. pylori* strains, a segment of *vacA* gene, encoding C-terminal domain of the protoxin and a segment situated near the N-end are strongly conservative. On the other hand, the so-called middle region (region m) and the fragment encoding a protein signal sequence present high level of variability. The signal sequence (s) encodes signal peptide, which precedes the sequence

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of the particle of the main toxin and it is the part of the apparatus that transports the molecules of vacuolating cytotoxin of 87-95 kDa. The middle region encodes the protein of the cytotoxin [4,5].

There are two main types of signal sequence: *s1* (subtype *s1a*, *s1b*, *s1c*) and *s2*, and two of middle region: *m1* and *m2* (subtype *m2a* and *m2b*) [6]. The existence of various genotypes of s and m regions enables the formation of numerous genotypic combinations of *vacA* gene.

Toxicity studies on *H. pylori* strains performed *in vitro* on cell-lines have shown that non-cytotoxic (Tox-) strains lack a short fragment in the s region in (Tox-) strains. This fragment influenced the absence of the splitting of vacuolating toxin into two subunits and caused the changes of its activity. This in turn caused loss of the ability to induce the formation of acidic vacuole in the infected cell [7]. The type *m1* alleles are observed mainly in *H. pylori* strains type (Tox+), causing the vacuolization of epithelial cells, while type *m2* alleles are found in non-toxic strains (Tox-). The presence of *s1a* or *s1b* alleles in s region is characteristic for (Tox+) strains while *s2* occurs in (Tox-) strains.

The studies in vitro have shown that the structure of *vacA* gene determined its cytotoxic activity for HeLa cells. The type *s1a* is more active than type *s1b*. As far as type *s2* was concerned, its cytotoxic activity was not observed. The type *m1* is more active than type *m2* [3,8]. It was observed that *H. pylori* strains of *s1m1* allele profile were characterized by a high production of active cytotoxin and caused the vacuolization of epithelial cells to a greater extent. Thus, higher level of pathomorphological changes was suspected to occur in the gastric mucosa in patients infected with those strains. The strains of *s1bm1* profile showed slightly lower levels of produced cytotoxin, in case of *s1m2* - the levels were still lower, and *s2m2* system showed indeterminate levels of produced cytotoxin [8].

It appears that, allele *s2* is defective and induces the secretion of small amounts of the cytotoxin from the bacterial cell, and therefore leads to the mild course of infection [9]. The strains of *s2m2* profile do not present toxic activity *in vitro* and do not cause the vacuolization of HeLa cells [10]. *H. pylori* strains of *s1m2* or *s2m1* gene allele combinations appear due to recombination of bacterial genetic material [3].

There are also certain relationships found between presence and expression of *vacA* gene and cagA pathogenicity island. In most cases, the presence of cagA pathogenecity island has been correlated with the presence of *vacA s1* gene. On the other hand, all strains without cagA expression show the presence of *s2* allele of *vacA* gene [11].

The aim of the present study was to evaluate frequency of *vacA* gene alleles in *Helicobacter pylori* strains in children and adults in Podlaskie region.

Material and methods

The examined group comprised 68 subjects (38 children and 30 adults, inhabitants of the Podlasie province) positive for IgG antibodies against *H. pylori*. The age of children ranged from 2 to 8 years (mean 12.6 yrs) and adults from 23 to 56 years (mean 39.3 yrs). Anti-*H. pylori* level in children ranged from 25 to 181 U/ml (mean 110 U/ml) and in their parents from 25 to 168 U/ml (mean 117 U/ml). As far as the dwelling place was concerned, the study regarded 38 subjects from the urban area (22 children and 16 parents) and 30 patients from the rural area (16 children and 14 parents). Recom Well Helicobacter IgG (Mikrogen GmbH) kit was used for detection and direct identification of anti-*H. pylori* IgG antibodies in human serum. The test used in the examination is a screening test based on ELISA method. IgG concentration against *H. pylori* >24 U/ml was considered the positive result.

All the patients positive for IgG antibodies against *H. pylori* and accompanying dyspeptic symptoms underwent gastroscopy. Endoscopic examination of the upper part of the digestive tract, enabled evaluation of macroscopic changes according to Sydney classification. The specimens for the traumatic test were collected from the antrum. Two specimens of the mucous membrane from the prepyloric part, gastric corpus, and areas of pathological lesions were taken for histopathological examination. The histopathological evaluation of bioptates was conducted using the Sydney System and intensification of the features of inflammatory mucosa was rated on a four-point scale (0-1-2-3).

Material for genetic examination covered bioptates sections of the corpus and antrum collected during gastroscopy and *H. pylori* DNA was isolated. The genotype of 70 strains of *H. pylori*, including *vacA* genotype of 38 strains of *H. pylori* isolated from children and the genotype of 32 strains of *H. pylori* isolated from adults, was evaluated in 68 paraffin sections.

DNA for genetic studies was isolated from three to four 3 m thick paraffin embedded bioptate sections of the corpus and antrum collected during gastroscopy, after paraffin removing with xylene and ethanol. DNA isolation was performed with a commercial Sherlock AX kit provided by A&A Biotechnology (Gdansk, Poland) according to manager protocol. Isolated DNA was resolved in 50 µl of TE buffer (pH=8.0) and stored at -20°C until use. In order to confirm absence of PCR inhibitors, amplification of a part of a human K- RAS gene was performed for each analyzed DNA solution (5'-ACTGAATATAAACTTGTGGTAGTTGGACCT-3' - with primers forward K1 and reverse K2 5'-TCAAAGAATGGTCCTGGACC-3').

For *vacA* genotyping signal region *s1*/*s2* alleles and midregion *m1*/*m2* alleles of the gene were determined by nested or semi-nested PCR methods with sets of primers described by Koehler et al in 2003 [12]. Semi-nested PCR reaction generated products 120 bp and 150 bp long for *s1* and *s2* alleles, respectively. Nested-PCR amplification of *vacA* gene middle regions *m1* and *m2* produced products 301 and 102 bp long, respectively.

The PCR reaction mix was the same for the all amplification reactions and contained 10 mM Tris-HCL, pH=8.3, 50 mM KCL, 1.1 mM $MgCl_2$, 0.01% gelatin, 0.5 M each of forward and reverse primers (of external or internal pairs according to amplified allele), 200 µM each of four dNTPs, 5 ml and 2.5 Units of REDTaq DNA Polymerase (Sigma, USA) in a total volume of 25 µl. After 5 minutes of target DNA denaturation at 94°C, 40 cycles of amplification (each consisted of denaturation at 95°C for 1 minute, primers annealing at 58°C for 1 minute and primers extension at 72 for 1 minute) followed by 7 minutes extension of all products were performed in a thermal cycler (Eppendorf, Germany). Aliquots of 10 µl of the second PCR products were analyzed by electrophoresis in 3% agarose TBE gel and bromide ethidium staining. The alleles of the s- and m- regions of the *vacA* gene were determined on the basis of the size of PCR products.

The statistical significance of differences between the groups was tested by χ^2 test. $P \leq 0.05$ were considered statistically significant.

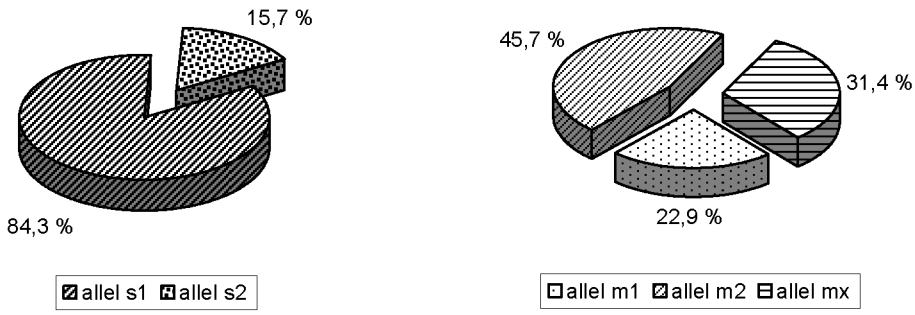

Fig. 1. Frequency of *H. pylori* gene *vacA* middle region (m) and signal sequence (s) in total group of patients.

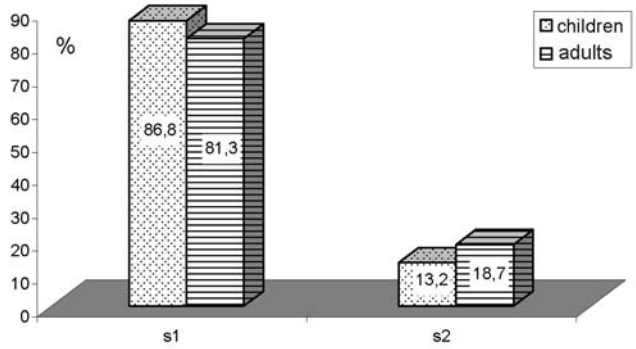

Fig. 2. The frequency of alleles of the s region in children and adults.

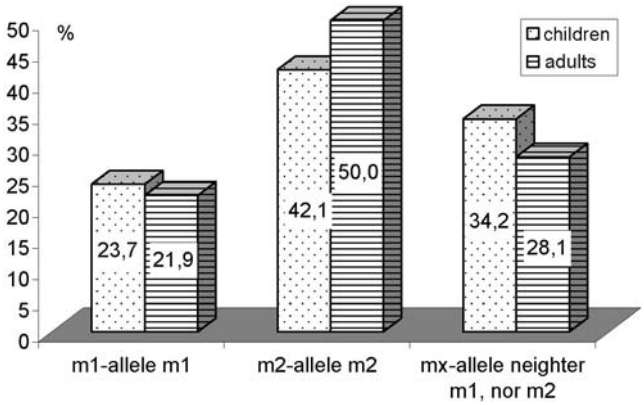

Fig. 3. The frequency of *H. pylori vacA* gene middle region alleles in infected children and adults.

Results

The primers on the allele *s1*, *s2* and *m1*, *m2* used in the study enabled the differentiation and characteristics of allele of s region and m region in *vacA* genotype isolated from examined *H. pylori* strains.

Human K-RAS gene PCR amplification revealed sufficient quality of all analyzed DNA solutions (data not shown).

The most frequently observed *H. pylori vacA* gene signal region allele, both in children and adults, was the *s1* allele. Allele *s1* of the region of the examined gene was determined in 59 out of 68 (84.3%) DNA isolates, whereas allele *s2* in 11 out of 68 (15.7%) DNA isolates. Among children analyzed: 33 out of 38 (86.8%) were infected with *vacA s1* allele *H. pylori* strains and 5 out of 38 (13.2%) had *s2* allele form of bacteria. In adult patients: 26 out of 32 (81.3%) were infected with *vacA s1* allele *H. pylori* strains, and 6 out of 32 (18.7%) had *s2* allele form of bacteria. There were no statistically significant differences found in particular alleles frequencies in the examined children and parents. Fig. 1 presents the results of electrophoretic examination of PCR products for *H. pylori* gene *vacA* signal region in DNA extracted from four patients.

H. pylori vacA gene *m1* or *m2* alleles of midregion were found in 48 out of 68 (70.6%) of analyzed DNA. Among them *m1* allele was shown in 16 out of 68 (23.5%) samples and allele *m2* in 32 out of 68 (47.1%) samples. In 22 out of 68 (32.3%) DNA extracts neither *m1*, nor *m2* allele was amplified. We called the undetermined allele of *vacA* gene midregion mx. The results of midregion *vacA* gene alleles are presented in Fig. 2.

We did not observe any statistically significant differences as far as alleles *s1* and *s2* occurrence was concerned in relation to the dwelling place; both in the rural area (77.4%) and the urban area (89.7%) the presence of allele *s1* was more frequent.

The percentage of isolated alleles *m1* and *m2* in the genetic material of the examined patients with *H. pylori* infection varied in relation to the dwelling place (data statistically significant).

The allele *m2* occurred more often in inhabitants from the urban area (data statistically significant, $p<0.05$) whereas the allele *m1* was more frequently observed in patients living in the rural area ($p\leq0.05$).

The presence of allele *s1* was observed in 84.3% of the examined *H. pylori* strains, allele *s2* - in 15.7%, allele *m1* - in 22.9% while *m2* was observed in 45.7%.

In 22 isolated *H. pylori* strains (31.4%), allele was not determined using the primers on allele *m1* and *m2* (the undetermined region part was marked in own studies as mx) (Fig. 1).

Allele *m1* of *vacA* gene amplified in the analysis of PCR product with the use of the electrophoretic technique in agarose gel was presented in Fig. 4.

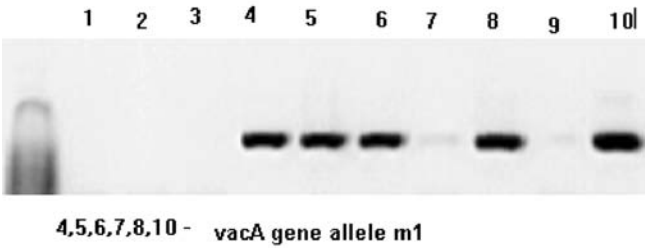

Fig. 4. Allele *m1* of *vacA* gene detected by PCR and subsequent agarose gel electrophoresis.

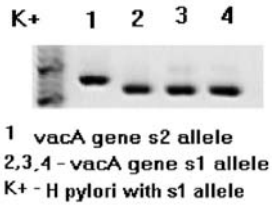

Fig. 5. The presence of allele *s1* and allele *s2* of *H. pylori vacA* gene - the analysis of PCR product with the use of the electrophoretic technique in agarose gel.

The presence of allele *s1* and allele *s2* of *H. pylori vacA* gene in the infected parent (P.P.), observed during the analysis of PCR product with the use of the electrophoretic technique in agarose gel, was presented in Fig. 5.

The most frequently observed allele of gene *vacA* signal region, both in children and adults, was the allele *s1* (86.8% and 81.3%, respectively); the allele *s2* was isolated in 13.2% of children and 18.7% of parents. The percentage of alleles of the s region and m region in children and adults was presented in Fig. 2 and Fig. 3.

Discussion

The examination of *vacA* genotype showed that the prevalence of *H. pylori* strains containing alleles *s1* or *s2* is various in different parts of the world.

The studies concerning the geographic distribution of *H. pylori* strains with subtypes of the allele *s1* (*s1a*, *s1b*, *s1c*) revealed 89% of the subtype *s1a* in the Northern and Eastern Europe. The subtype *s1a* occurs most frequently in France and Italy while Spain and Portugal show that 89% of strains contain the subtype *s1b*. On the other hand, 79.61% of examined *H. pylori* strains contain the subtype *s1a* in Germany [13].

Both *s1a* and *s1b* alleles present almost identical percentage (65-100%) in North America while Middle and South America reveal the subtype *s1b* in all *H. pylori* strains [14]. The subtype *s1c* is observed in 77% of *H. pylori* strains in Eastern Asia and the allele *s1a* is the most frequently occurring subtype in *H. pylori* strains in Japan [15].

Many reports have revealed the differentiation in prevalence of midregion alleles in the examined populations.

The region *m1* was more often observed (>80%) in South America, Portugal and Spain [3,8,13]. In China, the prevailing strain is that containing region *m2* [15] while in Japan the strains with region *m1* are more frequent [13,14].

The percentage of alleles *m1* and *m2*a occurrence is equal in Northern Europe and North America. The allele *m1* is more common (86.2%) in the Iberian Peninsula and Middle and South America than *m2* (13.8%). The subtype *m2*b was observed solely among *s1c* strains in eastern Asia [16].

In the studies conducted in Poland, the presence of *s1* allele was presented in 83.4% of *H. pylori* strains [17]. The allele *s2* was present in 20.2%, *m1* in 33.3%, and *m2* in 80.9% of isolated *H. pylori* strains.

We evaluated *vacA* genotype of 70 *H. pylori* strains and it turned out that the most frequently occurring allele of the region encoding the signal was the allele *s1*, which appeared in 84.3% of the examined group (86.8% in children and 81.3% in adults). We did not observe any statistically significant differences as far as *s1* and *s2* alleles were concerned in relation to the dwelling place.

The allele *m2* of the midregion was the one most frequently observed in our studies (42.1% in children and 50% in adults). It was also more frequently found in inhabitants of the urban areas.

The allele *m1* occurred in 23.7% of children, 21.9% of adults and was statistically more frequent in *vacA* genotype of *H. pylori* strains in patients from the rural areas.

Despite using the primers on *m1* and *m2*, we could not confirm their presence in 34.2% of examined children and 28.1% of adults.

It seems that those *H. pylori* strains contain an undetermined allele m subtype (mx in own studies). The primers *m1*F1 and *m1*R1 on *m1* area and *m2*F1 and *m2*R1 on *m2* area used in the study did not detect any determined area of a given *H. pylori* strain genome.

Strobel [11], in his studies conducted in adult patients in Germany, isolated *m1* and *m2* alleles in 81.5% of examined bacteria strains. The rest 18.5% of strains analyzed with the primers used on *m1* and *m2* typing, did not show any PCR products. Further studies revealed that places of binding specific for *m2* were not present and the place of VA3-R primer on *m1* was changed. The analysis of DNA sequence revealed that mx region showed higher homology with *m1* strains (88%) than *m2* (74%). Thus, the researchers suggested that mx is a new *m1* subtype (*m1a*), which is observed only in isolates of strains occurring in Europe.

Doorn *et al.* [6], while evaluating *vacA* genotype of *H. pylori* strains isolated from patients from various parts of the world detected 3 different types of midregion alleles (*m1*, *m2a*, and *m2b*).

Pan [15] also described a variant of midregion allele, observed very rarely, in which a midregion allele is a hybrid and has the same structure as *m1* allele in its proximal part and as *m2* allele in its distal part.

In multicenter studies, conducted by Suerbaum [18], it was suggested that in countries, where both alleles (*m1* and *m2*) are present in the same percentage in *H. pylori* infected populations, alleles *m1* and *m2* undergo recombination.

H. pylori virulence is conditioned by its genetic structure and the proteins encoded by particular genes participate in the process of gastric mucositis in children and adults.

Conclusions

We did not detect statistically significant differences in relation to the occurrence of particular alleles in the examined children and adults. We did not find statistically significant differences of *s1* and *s2* alleles occurrence in relation to the dwelling place. The percentage of isolated *m1* and *m2* alleles in the examined genetic material differed depending on the dwelling place (data statistically significant).

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