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Original articles

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EPIDEMIOLOGY OF cagA/vacA GENES IN *H. PYLORI* ISOLATED FROM CHILDREN AND ADULTS IN POLAND

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Serious lesions in gastric mucosa seem to be a result of infection with more pathogenic *H. pylori* strains. It was shown that two different proteins: CagA and VacA are the pathogenicity markers of *H. pylori*. CagA/VacA profile is associated with high intensity of inflammation and development of peptic ulcer disease. Presence of CagA protein depends on possession by a strain of cagA gene. VacA protein is expressed in every strain, but particular alleles of vacA gene are responsible for different levels of cytotoxin production. We estimated frequency of the cagA gene and particular alleles of vacA gene in 84 *H. pylori* strains by PCR method. Eighty percent of children and 72.4% of adults were infected with cagA(+) *H. pylori* strains. Presence of cagA gene was correlated with active gastritis in 60% of infected children and adults. Majority of *H. pylori* strains represented s1m2 and s1m1 vacA alleles' combinations that are responsible for high and medium level of cytotoxin production. Our data may suggest high risk of development of serious consequences of *H. pylori* infection especially in children treated unsuccessfully.

Key words: *cagA, vacA, pathogenicity island, PCR*

INTRODUCTION

H. pylori, microaerophilic Gram negative spiral bacterium, is implicated as the main etiologic agent of chronic active gastritis and peptic ulcer (1—3). It has been shown that active gastritis is a risk factor for peptic ulcer disease (4—6) and gastric cancer (3, 7, 8,). However, most of the infected people are asymptomatic (9). Only in small proportion of infected subjects gastritis can promote to non-ulcer dyspepsia, duodenum or gastric ulcers or to atrophic gastritis and atrophy of gastric mucosa is recognised as a first step leading to cancer or MALT-lymphoma (7, 9, 10). *H. pylori* is the most common chronic bacterial infection in the world population with much higher incidence in

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developing countries (9, 11). *H. pylori* mechanisms involved in gastroduodenal pathology are still not clear. A lot of properties were characterised, that allow these bacteria to survive in acid environment, multiply and colonise the gastric niche. Strains of *H. pylori* represent high degree of genetic diversity but all phenotypic features are very conservative. The synthesis of vacuolating toxin-VacA, responsible for intensification of inflammatory process and development of peptic ulcer disease can differ in individual *H. pylori* isolates (12). Quantity of toxin produced *in vitro*, which is able to induce cell vacuolisation depends on vacA gene structure. Presence of three different alleles for signal sequence (s1, s1a, s1b) and two alleles for middle region (m1, m2) allow to distinguish 6 possible combinations. Only one of them is present in vacA gene resulting in characteristic information. *H. pylori* strains with s1m1 alleles produce *in vitro* higher levels of toxin in comparison to strains with s1m2. Strains with s2m2 alleles have lowest vacuolatin toxin activity (13).

It was shown that the *H. pylori* vacA(+) can carry cagA gene encoding cytotoxin-associated protein. The gene is localised in different part of the *H. pylori* genome then vacA. This surface-associated protein of molecular weight 120—140 KDa does not have property of cytotoxin but is strongly immunogenic. According to Atherton *et al.* presence of cagA gene is connected with coexistence of s1 type of signal sequence in vacA gene. There is a suggestion of connection between vacA genotype and risk of peptic ulcer disease (9, 13). Sera of 60—76% patients with gastritis (14, 15), 80—100% patients with peptic ulcer disease (9) and most patients with gastric cancer had antibodies to CagA protein (16). Detection of cagA gene and vacA alleles may be recognised as risk factors for severe diseases (17, 18). The strict correlation between presence of CagA protein in *H. pylori* strains and a rate of PMN and lymphocyte's infiltration in antrum, and the presence of peptic ulcer or gastric carcinoma has been shown in adults (16, 18). Clinical and histopathological symptoms of *H. pylori* infection in children are often intermediate. The correlation between cagA/vacA alleles of *H. pylori* and severity of infection in children has not been found and elucidated. The aim of this work was to find the frequency of presence of cagA gene among the strains of *H. pylori* isolated from children and adults and to determine the frequency of different alleles of vacA gene in *H. pylori* as risk factors of serious consequences of infection.

MATERIAL AND METHODS

Bacterial strains

Gastric biopsies taken from patients during gastroendoscopy after homogenization were cultured on Wilkin's Chalgren medium with horse blood (7%), vancomycin (10 mg/l), trimetoprim (5 mg/l), cefsulodin (5 mg/l) and cycloheximid (100 mg/l). Plates were incubated in microaerophilic

atmosphere with 5-8% CO_2 at 37°C for 5—7 days. Species identification was based on colony and cell morphology as well as on biochemical features. The enzymatic activity of oxidase, catalase and urease was identified by use of API CAMP and *Rapidec pylori* (bioMerieux) tests.

Histopathology

Several layers of cut biopsy were analysed after hematoxylin and eosin staining to grade severity of gastritis. Histology examination was performed in the Department of Pathomorphology, Children's Memorial Health Institute.

DNA isolation from bacterial strains

Colonies of *H. pylori* from one plate were scrapped and washed twice in TE buffer (10mM Tris HCl, 1 mM EDTA pH 8.0) and then resuspended in 0.5 ml PBS. After centrifugation the pellet was subjected to lysis in 0.5 ml STE buffer (150 mM NaCl, 10 mM Tris HCl, 1 mM EDTA), containing lysosyme (Sigma) to final concentration 3.5 mg/ml. Lysate was incubated 30 minutes at 37°C and after addding of proteinase K (Sigma) in concentration 200 g/ml 30 min. Lysis was prolonged to 2 h at 37°C after adding 1% of SDS. Then RNA-asis was added (10 mg/ml) and the sample was incubated 1 h at 37°C. DNA was extracted twice with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and twice with chloroform: isoamyl alcohol (24:1). DNA was precipitated with 3M sodium acetate, pH 5.5 and absolute ethanol (2). After 1h incubation at −70°C samples were centrifuged 12000 c/min for 30 min at 4°C. Then DNA pellet was washed in 70% ethyl alcohol and dried (2). DNA was resuspended in 40 µl of TE buffer (10 mM Tris, 1 mM EDTA). Quality and quantity of DNA were spectrophotometrically determined by measurement of absorbency at 260 nm and 280 nm (spectrophotometer Becman DV-62) and by electrophoresis of DNA samples on 1% agarose gel in comparison with diluted preparations of phage DNA.

Detection of cagA gene by PCR

The chosen primers:

Luni 1- 5'-ACATTTTGGCTAAATAAACGCTG-3'

and

L cag1- 5'-TCTCCATGTTGCCATTATGCT-3'

determined for amplification of 360 bp fragment of left end of the whole pathogenicity island that is the synonyms with cagA gene.

PCR reaction was carried out in volume of 50 µl in Gen Amp 9600 thermocycler (Perkin Elmer). The mixture contained incubation buffer 10× (10 mM TrisHCl pH 8.3; 50 mM KCl; 2.5 mM $MgCl_2$; 0.001% gelatine); 4 deoxyribonucleotides, 100 M each; primers, 50 pM each; Taq polymerase (Boehringer)-2 U and 0.5-1 µg of target DNA.

Primers:

Luni1- 5'ACATTTTGGCTAAATAAACGCTG-3'

and

Rnew1-5'TCATGCGAGCGGCGATGTG-3'

determined for amplification of 360 bp fragment of DNA — "empty place" of the whole pathogenicity island. The same quantities of mixture components were used for PCR amplification. Sequence of primers was kindly provided by Prof. D.E. Berg (Washington University, St. Louis, USA).

Conditions of PCR reaction: denaturation — 94°C, 4 min; followed by 35 cycles of denaturation (94°C, 1 min), annealing (57°C, 1 min) and extension (72°C, 1 min); and final extension —72°C, 5min.

Controls: positive — amplification of DNA isolated from *H. pylori* cagA(+) NTCT26695 strain, provided by Prof. D.E. Berg (Washington University in St. Louis, USA).

PCR with water as a target was a negative control.

Amplification products were analysed by electrophoresis in 2% agarose gel stained with ethidium bromide (0.5 μg/ml) under UV illumination (Pharmacia).

Detection of vacA alleles

Following sequences of primers were used for determining presence or lack of specific alleles in each *H. pylori* strain (13):

m1: VA3-F 5'-GGTCAAAATGCGGTCATGG-3',

VA3-R 5'-CCATTGGTACCTGTAGAAAC-3',

designed for amplification of 290 bp fragment.

m2: VA4-F 5'-GGAGCCCAGGAAACATTG-3',

VA4-R 5'-CATAACTAGCGCCTTGCAC-3',

designed for amplification of 352 bp fragment.

s1: VA1-Fc 5'- ATGGAAATACAACAAACACAC-3',

VA1-Rc 5'-CTGCTTGAATGCGCCAAAC-3',

designed for amplification of 259 bp fragment or characteristic for **s2** allele 286 bp fragment .

s1a: SS1-Fe 5' GTCAGCATCACACCGCAAG-3' and VA1-Rc,

designed for amplification of 190 bp fragment.

s1b: SS3-Fe 5' AGCGCCATACCGCAAGAG-3' and VA1-Rc,

designed for amplification of 187 bp fragment.

s2: SS2-Fe- 5' GCTAACACGCCAAATGATCC-3' and VA1-Rc,

designed for amplification of 199 bp fragment.

For each pair of starters the same conditions were used: denaturation (94°C, 4 min); followed by 35 cycles of denaturation (94°C, 1 min), annealing (57°C, 1 min), extension (72°C, 1min); and final extension —72°C, 5 min. Reaction mixture contained incubation buffer 10× (10 mM TrisHCl pH 8.3; 50 mM KCl; 2.5 mM $MgCl_2$; 0.001% gelatine); 4 deoxyribonucleotides, 100 μM each; primers, 50 pM each; Taq polymerase (Boehringer)- 2 U and 0.5-1 μg of target DNA. Reaction was carried out in volume of 50 μl in Gen Amp 9600 thermocycler (Perkin Elmer).

RESULTS

Frequency of isolation of cagA(+) H. pylori strains

Among 84 strains (55 isolated from children and 29 from adults) presence or lack of cagA gene was determined by amplification of left end of the whole pathogenicity island (*Fig. 1*).

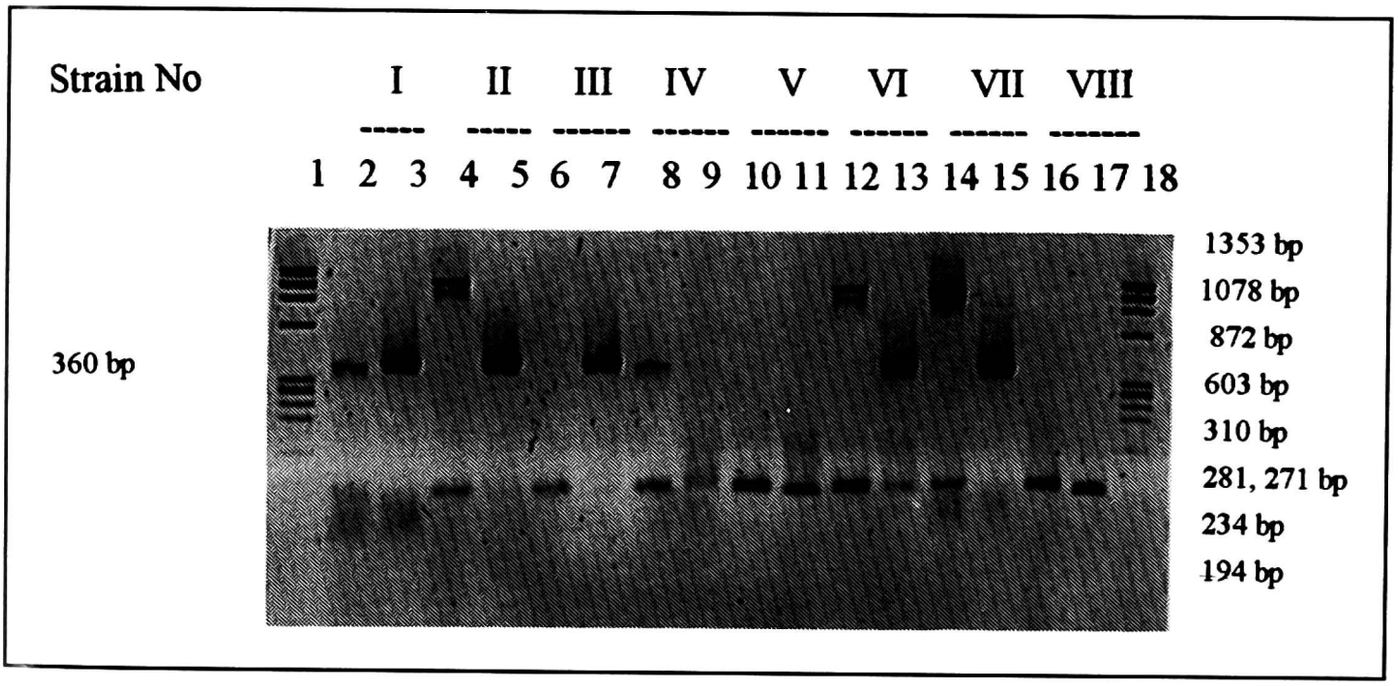

Fig. 1. Electrophoresis of 360 bp amplified products of left end of pathogenicity island (primers Luni1 i LcagA) and of 360 bp amplified fragment of "empty side" for pathogenicity island (primers Luni1 i Rnew1). 1, 18- molecular marker ϕX 174 RFI DNA/HaeIII (Amersham), 2-18 — tested samples, where odd indicates result of amplification for "empty side" and even indicates result of amplification for pathogenicity island.

The results of presence of cagA gene in tested population of *H. pylori* strains isolated from children and adults are shown in *Tables 1* and *2*.

Table 1. Frequency of cagA gene detected by PCR method among 55 *H. pylori* strains isolated from children with different histopathology pictures.

	Active gastritis	Non active gastritis	Normal state of mucosa	Duodenitis	Ulcer duodeni + Gastritis	Duodenitis + Gastritis
cagA(+)	33(60.0%)	5(9.1%)	3(5.5%)	1(1.8%)	1(1.8%)	1(1.8%)
cagA(−)	6(10.9%)	1(1.8%)	3(5.5%)	—	—	—
cagA(+)/(−)	1(1.8%)	—	—	—	—	—

Table 2. Frequency of cagA gene detected by PCR method among 29 *H. pylori* strains isolated from adults in different histopathologic pictures.

	Active gastritis	Non active gastritis	Normal state of mucosa	Duodenitis	Duodenitis + Gastritis
cagA(+)	17(58.6%)	—	2(6.9%)	1(3.45%)	1(3.45%)
cagA(−)	3(10.35%)	1(3.45%)	1(3.45%)	—	—
cagA(+)/(−)	3(10.35%)	—	—	—	—

CagA(+) *H. pylori* strains were isolated from 60% of children with active gastritis. Three strains isolated from children with duodenitis or ulcer duodeni had also cagA gene. Some (3) strains isolated from normal gastric mucosa were cagA(+). Only 12.7% of strains isolated from gastritis (active or non active) were cagA(−). One child was infected by mixture of cagA(+) and cagA(−) subpopulations. As much as 58.6% of strains isolated from adults with active gastritis were cagA(+). *H. pylori* strains without cagA gene were isolated from 4 adults with gastritis and from one patient with normal gastric mucosa. It should be stressed that the strains isolated from patients with duodenitis or ulcer duodeni cases were cagA(+). Mixed infections cagA(+)/(−) were identified in 3 adult patients.

Correlation between presence of cagA gene and types of vacA alleles

Presence or lack of amplified products of each vacA allele allowed us to determine the s/m combination in genomes of tested strains. Examples of detected amplified products are shown in *Fig. 2* and *Fig. 3*.

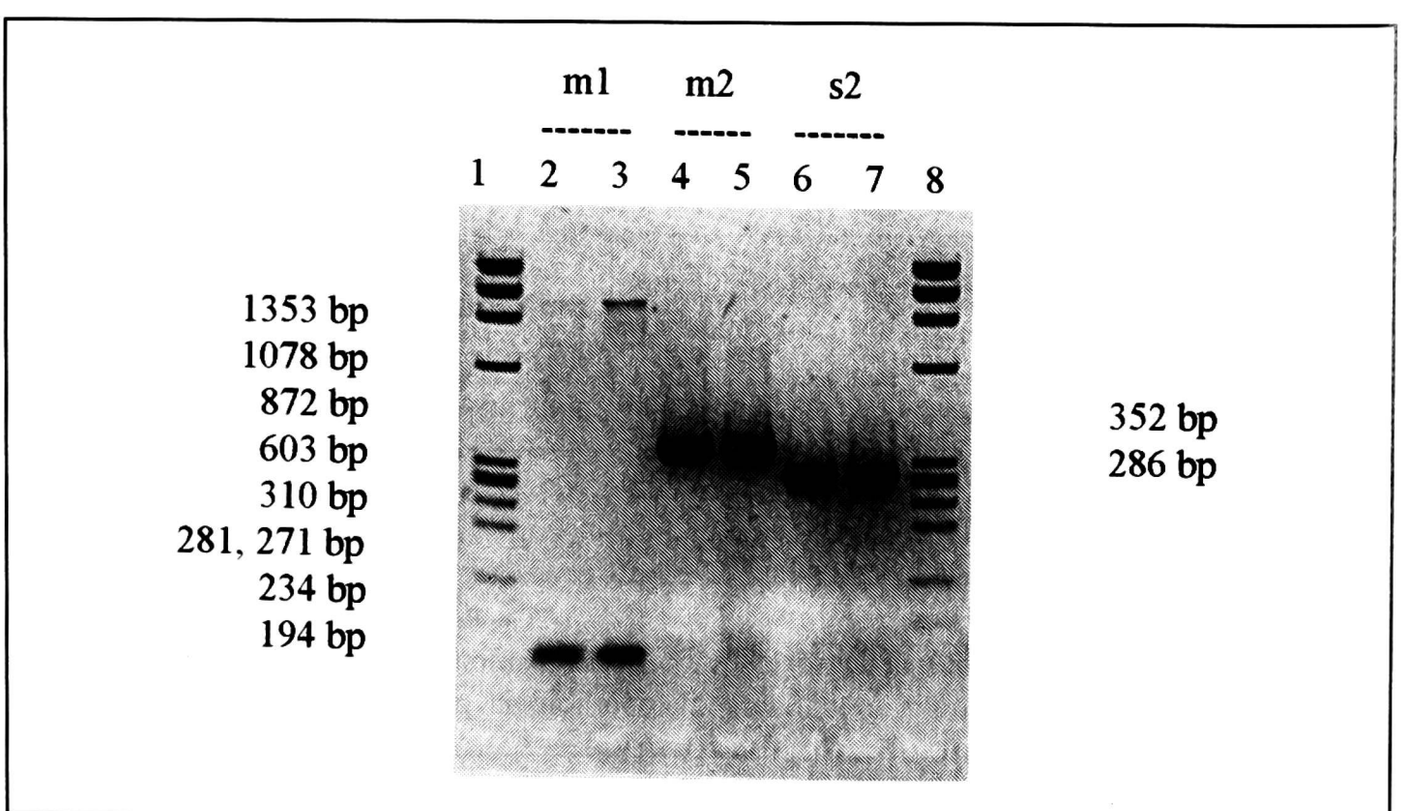

Fig. 2. Detection of amplified products on agarose gel : (2-3) primers VA3-F i Va3-R used for detection of m1 allele (290 bp.); (4-5) primers Va4-F i VA4-R used for detection of m2 allele (352 bp.); (6-7) primers VA1-Fc i VA1-Rc used for detection of s2 allele (286 bp.). Tested strain was isolated from one patient before (rows: 2, 4, 6) and after treatment (rows 3, 5, 7); 1,8- molecular marker ϕX 174 RFI DNA/HaeIII (Amersham).

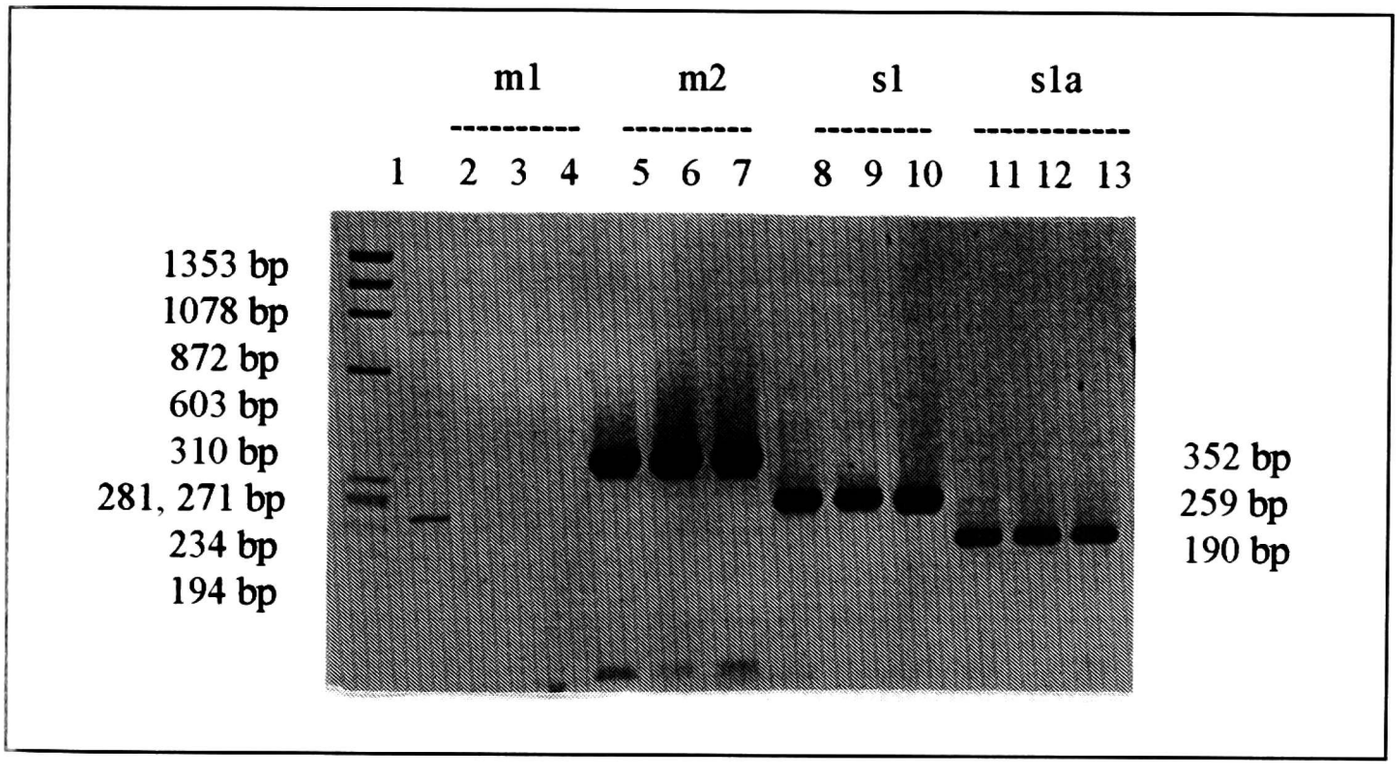

Fig. 3. Electrophoresis of amplified products by application of: (2-4) primersVA3-F i VA3-R used for PCR detection of m1 allele (290 bp.); (5-7)primers VA4-F i VA4-F used for detection of m2 allele (352 bp.), (8-10) primers Va1-Fc i VA1Rc used for detection of s1 allele (259 bp.) and (11-13) primers SS1-Fe i SS2-Fe used for detection of s1a allele (190 bp.). Presented strain was isolated from child before (rows 2, 5, 8, 11), and after treatment and (rows 3, 6, 9, 12) from child father (rows: 4, 7, 10, 13); 1 — molecular marker ϕX 174 RFI DNA/HaeIII (Amersham).

Table 3. Frequency of different alleles of vacA gene in correlation to presence of cagA gene in 84 *H. pylori* strains isolated from children and adults.

Allele of vacA gene	Number of strains	Number of cagA(+) strains	Number of cagA(−) strains
s1	70 (83.4%)	66 (94.3%)	4 (5.7%)
s2	17 (20.2%)	5 (29.4%)	12 (70.6%)
m1	28 (33.3%)	28 (100%)	—
m2	68 (80.9%)	53 (77.9%)	15 (22.1%)

Distribution of alleles of vacA gene in the strains of *H. pylori* tested are presented in *Table 3*. It was shown that the s1 sequence connected with the presence of cagA gene was present in most tested strains (94.3%). This allele was more frequently seen among all strains analysed (83.4%). Allele s2 was identified in 12 cagA(−) *H. pylori* strains and 5 cagA(+) strains. Thirty-three percent of tested strains had m1 allele and all of them were cagA(+). Middle region type 2 was more frequent, 80.9% of strains had this sequence and 77.9% of them were cagA(+).

Frequency of s/m combinations

The distribution of different s/m combinations among tested strains is shown in *Table 4*. The most frequent combination was s1/m2 that was observed in (50.0%) of strains and 92.9% of them were cagA(+). All 16 (19%) strains with s1m1 combination were cagA(+). Strains with s2m2 were seen in 15.5% of isolates and 84.6% of them were cagA(−). In 12 strains mixed subpopulations were noticed (9 strains of type: s1m1/m2 were cagA(+).

Table 4. Frequency of different combination's s/m of vacA gene among 84 tested *H. pylori* strains isolated from children and adults.

Combination s/m	Number of strains	Number of cagA(+) strains	Number of cagA(−) strains
s1m1	16(19%)	16(100%)	—
s1m2	42(50%)	39(92.9%)	3(7.1%)
s2m2	13(15.5%)	2(15.4%)	11(84.6%)
s1m1/m2	9(10.7%)	9(100%)	—
s1/s2m2	1(1.2%)	—	1(100%)
s2m1/m2	1(1.2%)	1(100%)	—
s1/s2/m1/m2	2(2.4%)	2(100%)	—

Allele s in our collection of strains is used for subtype s1a because subtype s1b was not observed among tested strains. Strains isolated from 11 children and 2 adults before and after therapy presented identical cagA/vacA profile of cagA/vacA as well as the strains isolated from children and their parents.

DISCUSSION

It is very intriguing why only a minority of infected patients develop serious gastroduodenal diseases. The reason for this phenomenon may partially be due to host differences, as well as the virulence factors of some bacterial strains necessary to induce serious lesions of gastric mucosa. Coexpression of vacuolating cytotoxin A (VacA) and cytotoxin-associated gene A (cagA) product (CagA) seems to be a phenotype predominantly present in the strains isolated from patients with peptic ulcer disease and gastric cancer (9). There are not many papers about *H. pylori* cagA(+) infection in children; however they suggest that the presence of CagA antigen is related to higher degree of

inflammation without presence of clinical symptoms. In most children infected with cagA(+) *H. pylori*, gastritis (but not ulcer disorders) was seen (19).

This work presents preliminary data on genetic character of *H. pylori* strains isolated from children and adults in Poland. As much as 53% of children and 70% of adults presenting clinical symptoms from upper gastrointestinal tract tested in our Institute had *H. pylori* infection. It was interesting to estimate how many infected children are at risk of development of serious disorders in case of inefficient treatment. The risk can be calculated on the basis of frequency of occurrence of virulence markers in the strains responsible for infection. One of virulence markers is cagA gene. It was shown that all genes within the region of "pathogenicity island" had an important role in pathogenesis of *H. pylori* infection (9). Inactivation of two open reading frames of two genes (picA, picB) in cagA region resulted in decrease of inflammatory activity by limited IL-8 secretion in gastric mucosa (20). Presence of cagA region in *H. pylori* tested strains was associated with active gastritis in 60.0% children and 58.6% adults. Independently from histopathological observation most children (80.0%) and adults (72.4%) were infected with cagA(+) *H. pylori* strains. These data can not be compared to patients with peptic ulcer because of limited number of groups examined in our study. Frequency of cagA(+) *H. pylori* infection varies in different geographical regions. In Western Europe and USA 63.0% of isolated strains were cagA(+), and 87.0% of them were isolated from patients with ulcer disease. However in Peru, Thailand and China most of *H. pylori* strains possess cagA region (9). It is interesting that only 30.0% of strains isolated from children in France are cagA-positive as well as 30.0% of *H. pylori* strains in North Africa are cagA(+) independently of histopathological changes (9). Our data suggest that the distribution of virulence markers (cagA) in strains of *H. pylori* isolated from children in Poland is higher than in the Western countries (30%) and lower than in the far East (e.g.Asia-almost 100% strains cagA(+). Presence of cagA was detected in 80% of cases with clinical symptoms. Data published by Atherton *et al.* (13) demonstrated the association between genotypes of infected strains and histopathological changes of gastric mucosa. Among mosaic alleles characterising signal sequence and middle region of vacA gene, s1m1 is responsible for the highest cytotoxin activity and is associated with peptic ulceration. We have tried to find the correlation between the s/m combinations and the presence the cagA region in the tested strains. Tested strains of *H. pylori* possessing s1, m1, m2, s2 alleles were cagA-positive: 94.6%, 100.0%, 77.9%, 29.4%, respectively. All strains with s1m1 alleles were cagA(+). As much as 92.9% of strains with s1m2 combination in vacA gene were cagA(+). Most strains with s2m2 alleles (84.6%) were cagA(−). We have found that two s2m2 strains were cagA(+) what is in contrast to the data published by Atherton *et al.* (13); his s2m2 strains were cagA(−). Thirteen patients tested in

our study had mixed infections caused by strains of *H. pylori* with more than one combination of alleles. Most of them (12/13) were cagA(+). Further work is needed to explain the nature of observed genetic variations. Data obtained in our population of *H. pylori* strains suggest the higher incidence of pathogenicity markers in comparison to the strains isolated in Western countries. Most of *H. pylori* strains had s1m2 and s1m1 combinations of alleles which are responsible for higher production of cytotoxin. It is interesting that only 13 *H. pylori* strains from 84 analysed showed the s2m2 profile responsible for the lowest cytotoxin activity. It is possible on the basis of cagA/vacA profile to deduce the risk of development of more serious consequences of *H. pylori* infection.

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