Original Article

Diagnostic Method For Detection Of Different Forms And Strains Of Helicobactor Pylori And Evaluation of its Eradication

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Key words: Helicobacter pylori, duodenal peptic ulcer, fingerprinting method

Abstract

The Aim of this study is the comparison of different diagnostic methods to detect different forms and strains of Helicobacter pylori (HP) isolated from gastric mucosa bioptats of the patients with duodenal and stomach peptic ulcer and the evaluation of HP eradication.

Methods: 28 patients with peptic duodenal ulcer were examined. HP in all patients bioptats was examined with urease test, histological method and polymerase chain reaction (PCR) before and after treatment. HP PCR-positive bioptats were examined with fingerprinting method PCR-RFLP.

Results: HP was revealed in all patients before treatment (the main blood IgG antibody's titerwas 36,7±16,6 u/ml). Electrophotogramms of amplificated restrict products obtained after restriction flaA fragments HP were used for fingerprinting in 7 out of 28 patients examined. Differences of restriction's pattern show the presence of 5 major HP strains in samples examined.

Introduction

Helicobacter pylori (HP) has important significance in peptic ulcer etiology. Helicobacter is not uniform. There are 19 species of Helicobacter. 8 species are persistent in gast. mucosa and 11 species are persistent in small intestinal mucosa. The man has only 7 species of Helicobacter: Helicobacter pylori, Helicobacter heilmannii, Helicobacter felis - in stomach mucosa; Helicobacter cinaedi, Helicobacter fennelliae, Helicobacter canis, Helicobacter pullorum are in small intestine mucosa. Each of specie including HP consists of a few strains [1]. All these strains may produce urease. Many tests for HP detection are based on high urease activity.

Determinants responsible for HP virulence are presented by vacA gene, which produces vacuolating cytotoxin; cytotoxin-associated gene cagA, gens of flagells flaA, flaB, genes that encode urease enzyme (ure A, B, C, D, E, F, G, H, I) as well as gene picB that has potential activity to induct IL-8 production by stomach epitheliumal cells [2]. HP strains that expressed specific proteins (vacuolating cytotoxin with 87 kD mass and proteinCagA with 120-128 kD mass and with clear antigen properties) encoded by gene cagA were demonstrated in some patients with peptic ulcer.

It is known, that HP strains have different resistance to the medicines, different adhesive specificity, different cytotoxin and protein CagA

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production, as well as different nucleotide genes (or its fragments), that encode particular HP proteins. FlaA, ureA, B, C genes are the most polymorphous. Genetic identification of HP strains now is possible due to the molecular diagnosis development. High sensitive and specific fingerprinting method was used with this aim during last years. There are several variants of fingerprinting: random amplified polymorphic DNA analysis (RAPD), restriction fragment length polymorphism analysis (RFLP), genomic restriction enzyme analysis (Genomic REA), restriction of specific PCR-amplified genes or PCR-based restriction fragment length polymorphism analysis (PCR-RFLP) [3]. All these methods are based on the intact DNA that was isolated from investigated biological material after preliminary bacteria's cultivation [4]. Restriction analyses of relatively small PCR products are used in PCR-RFLP method in contrast from RAPD, RFLP and Genomic REA methods. So theoretically it is possible to perform analysis of HP DNA, that was isolated from bioptats without preliminary bacteria's cultivation [5]. Solution of this problem is very important for scientific and practical objects:

To isolate and differentiate various HP strains directly from bioptats.

To find several HP strains in the single patient's bioptat.

To estimate resistance of individual HP strains to antihelicobacter therapy

To reveal dormant HP forms.

To reveal HP reinfection.

To our knowledge the experimental confirmation of such possibility was not shown.

The aim of this work - to evaluate diagnostic possibilities of various methods to detect different HP forms and strains and to evaluate efficiency of different HP treatment methods.

Material And Methods

28 patients with duodenal peptic ulcer were examined: 5 women 33-57 years old (the mean age X ±SD- 43,8 ±10,5) and 23 men 18-70 years old (mean age X ±SD- 43,6 ±15,7).

Often relapse of peptic ulcer were revealed in 14 patients (more than twice during a year). Esophagogastroscopy with biopsy from gastric antrum was performed with Pentax FG-29P (bioptats were kept in the temperature minus 200C). Preliminary selection of HP-positive patients with duodenal peptic ulcer was performed with quantitative spectrophotometric urease activity estimation [6,7]. Examined patients were divided in two groups depend on the method of treatment. The patients of the first group (n = 11) were given Omeprasol (Losec) 40 mg in a daily during 1 week and 20 mg in a daily during the next 3 weeks, Klarytromycine 250 mg three times daily - during 10 days.

The patients of the second group (n = 17) were given Pilorid 400 mg twice daily during 4 weeks and the same anti-HP treatment.

HP was examined in all patients before and after treatment with urease test, histological method and PCR [6,7]. The bioptats of all HP PCR-positive patients were examined with fingerprinting PCR-RFLP [3]. The base of PCR-RFLP is as follow: DNA HP is isolated from bioptat. Then amplification of gene (or its fragments) with high polymorphism was performed with PCR method and the use of proper primers. Amplification of flaA gen's fragment (size 1500 nucleotides pairs (1,5-kb)), that encodes HP flagellum protein synthesis, was performed in this work. Amplification products were exposed to electrophoresis in agarose gel for revealing of amplified fragment. Restrictase, that cuts the amplified fragment in definite sites of restriction were added at the last step.

Restrictase Hhal that cuts amplified fragment flaA gene in the places of guanin-cytosine-guanin-citosin (GCGC) siquence was used. Then electrophoresis of restriction products was performed. DNA isolation from bioptats and PCR with typospecific primers to HP use was performed with test-system sets of' Lagis" firm according to enclosed protocol.

DNA isolation from bioptats for fragment flaA gene amplification was performed with Insta Gene Matrix carrier of Bio-Rad firm. PCR was performed with next program:

Step 1: 94^{o}C - 2 min
Step 2: 94^{o}C- 30 sec
Step 3: 60^{o}C - 1 min
Step 4: 72^{o}C - 2 min
Step 5: 40 cycles steps 2-4
Step 6: 72^{o}C - 5 min

Oligonucleotides used as primers were 5'-ATGGCTTTTCAGGTCAATAC-3' and 5'-GCTTAAGATATTTTGTTGAACG-3'. Restriction was performed with restrictase Hhal ("Promega") according to the firm's protocol with working

-ATGGCTTTTCAGGTCAATAC-3' and 5'-GCTTAAGATATTTTGTTGAACG-3'. Restriction was performed with rest rictase Hhal ("Promega") according to the firm's protocol with working concentration 5 U/20 (1 test. 26 patients were repeated by examined (including esophagogastroduodenoscopy, HP definition with urease test, histological method and PCR) in 1 month and 24 patients were examined again in 6 months after treatment. Serological blood examination of HP antibodies presence (IgG) before treatment and after 1 and 6 month of treatment was performed as well in both patients groups.

Quantitative HP antibodies determination in the blood of all patients was performed by immunoenzyme method with special sets of firm "Lames" use (official distributor of firm "MEDAC Diagnostika" in Russia) according to enclosed protocol. Diagnostic threshold of antibodies titre was considered if ELISA results have been more 20 U/ml.

Results And Discussion

It is known, that HP infection leads to the production of special antibodies in the patients. Diagnostic titer of these antibodies is revealed on the second or third weeks from the beginning of HP infection. HP antibodies may long circulate in blood after HP eradication. All our patients had HP before treatment. The mean blood HP titer was 36,7 ± 16,6 un/ml. This fact revealed the active immune reaction to the HP antigen. The total HP eradication due to intensive anti-HP treatment during 1 month was demonstrated in the most patients (n =21). But 5 patients were HP-positive after anti-HP treatment. All patients were divided in two groups: HP PCR- positive and HP PCR- negative. The tendency to reduce of mean antibody level after 1 month of anti-HP treatment was demonstrated in both groups (Fig.l). But the diagnostic titer of antibodies was kept.

Preservation of antibodies diagnostic titer during 6 months after anti-HP treatment in PCR-positive patients had evidently connected with HP specific antigens presence in patient's organism and presumably was a consequence of HP in persisted dormant state [8]. But the subsequent reduce of HP antibodies level to the diagnostic titer in the PCR-negative patients was demonstrated (Fig.l). There is a tendency of reduction of HP antibodies level in PCR-negative patients with high determination coefficient (R2 -square of correlation coefficient = 0,997). The clear linear dependence of antibodies level in time after anti-HP treatment was shown.

Serological method reveals the specific HP antibodies presence that indicate contact and infection. The diagnostic titer of antibodies may be kept about 3 PCR-negative patients with high determination coefficient (R2 -square of correlation coefficient = 0,997). The clear linear dependence of antibodies level in time after anti-HP treatment was shown.

Serological method reveals the specific HP antibodies presence that indicate contact and infection. The diagnostic titer of antibodies may be kept about 3 months after the successful anti-HP treatment.

Peptic duodenal ulcer was revealed in 10 patients of the first group out of 11 patients with esophagogastroscopy. After treatment the ulcer was cicatrized in all 10 patients. Urease test, histology and PCR show absence of HP. 1 patient was included in this group since he had often relapses of disease and high degree of HP, but without ulcer under endoscopic examination. HP eradication after anti HP treatment was not demonstrated in this patient. Very high urease activity was found in this patient's gastric mucosa bioptat. This fact indicates the HP strain's resistance to klarytromycine and metronidazole.

The peptic duodenal ulcer was revealed in all 17 patients of the second group under endoscopic examination. The ulcer was cicatrized in all patients after treatment. HP eradication was demonstrated in 14 patients with PCR, urease test and histological method. HP eradication was not demonstrated in 3 patients. The high urease activity and positive PCR was revealed in 1 patient. It was possible the presence of HP strain in this case, that was resistant to the anti HP treatment. 2 patients had demonstrated HP PCR-positive, but HP urease activity in gastric mucosa bioptats of these patients was not revealed. This fact indicates possible HP transition to nonactive dormant forms.

The examination of 11 patients of the first group was repeated after the treatment of 1 month and clinical and endoscopic duodenal peptic ulcer remission was demonstrated in all patients. But the high urease activity and PCR-positive signal was again demonstrated in gastric mucosa bioptat of the patient with resistant HP strain (Fig.2a).

One more HP PCR-positive patient was revealed in this group, that indicates the possibility of reinfection.

Fifteen patients of the second group were investigated after period of 1 month. Duodenal peptic ulcer was revealed only in 1 patient with resistant HP strain. HP urease test, positive HP histological method and HP positive PCR were revealed in this patient. 2 other HP PCR-positive patients after anti HP treatment had revealed HP only by PCR (fig.2b).

9 patients of the first group and 15 patients of the second group were again examined after 6-month period. Duodenal peptic ulcer was not revealed in any

histological method and HP positive PCR were revealed in this patient. 2 other HP PCR-positive patients after anti HP treatment had revealed HP only by PCR (fig.2b).

Nine patients of the first group and 15 patients of the second group were again examined after 6-month period. Duodenal peptic ulcer was not revealed in any patients under esophagogastro-scopy. The patient of the first group, that didn't give reaction on anti HP treatment was not examined, since didn't come for control investigation. HP active form was revealed in one patient with urease test and PCR. Increase of PCR-positive patients without urease activity was demonstrated in(fig.2a) (n = 6). The same tendency was demonstrated in the patients of the second group in 6 months period after treatment (fig.2b).

These experiments demonstrated that urease test is not reliable test for evaluation of eradication efficiency, since HP elimination is not complete. HP might go into dormant form in some patients. The most sensitive and specific test of HP revealing is PCR. Urease test for HP revealing is advisable use only before eradication of HP treatment.

Increase of PCR HP-positive patients after 1 month and especially 6 month after treatment was demonstrated. It is possible suggest that reinfection as well as activation and formation of HP dormant forms took place. Identification of HP strains is necessary for solution of this problem.

The high sensitive and specific fingerprinting method is used in molecular diagnosis in the last years. Fingerprinting PCR-RFLP method was used in this work.

Electrophoregramms of amplified fragment flaA HP gene restriction products were demonstrated in 7 out of 28 patients. Differences of restriction's partten show the presence of 5 major HP strains in samples examined. Small amount of DNA patterns obtained from bioptats possibly were due to DNA HP destruction during the keeping (bioptats freezing and following throwing out) or by endogenic nucleases action and possible by these both factors. A loss of DNA amplification ability in long keeping bioptats that are repeated by used proofs this suggestion. Preliminary data indicate fingerprinting PCR-RFLP gives in some cases the opportunity to identify different HP strains directly in stomach mucosa bioptats. We consider that the following examinations with the aim to optimize the experiments conditions will increase its efficiency for typing of HP in mucosa bioptat. Fingerprinting PCR-RFLP may help discriminate the presence of resistant HP strains or dormant HP forms and to explain uneffectiveness of HP treatment. This method may lead to understanding of reinfection of HP and to support either infection with new HP strain or activation of its dormant forms.

References

1. Owen RJ. Helicobacter-species classification and identification. Brit. Med. Bul, 1998; 54(1): 17-30.
2. Zh. Ge and D.E. Taylor. Helicobacter pylori-

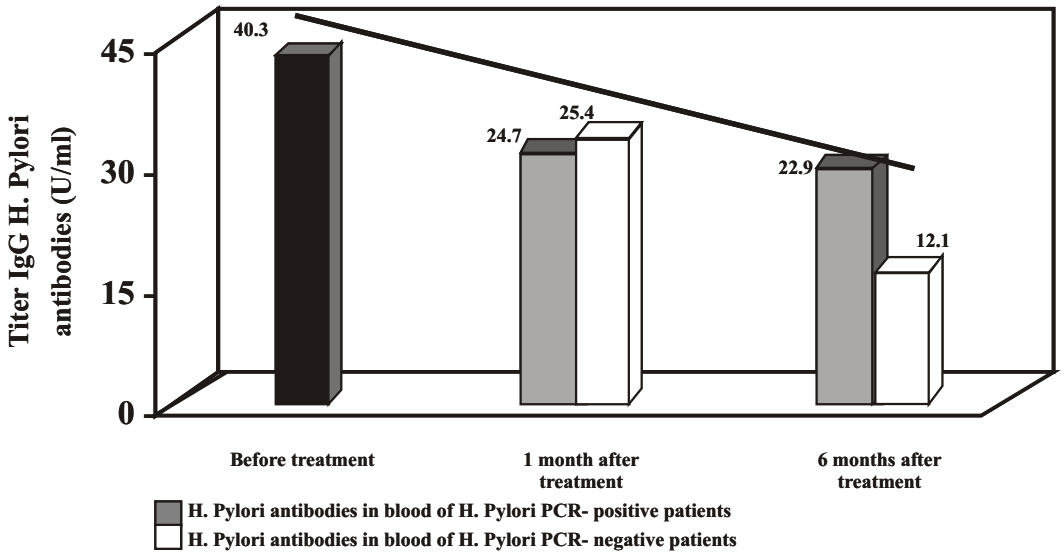

Fig -1
IgG H. Pylori antibodies level in blood of H. Pylori PCR - positive and H. Pylori PCR - negative patients after 1 and 6 months of anti H. Pylori treatment (ELISA)

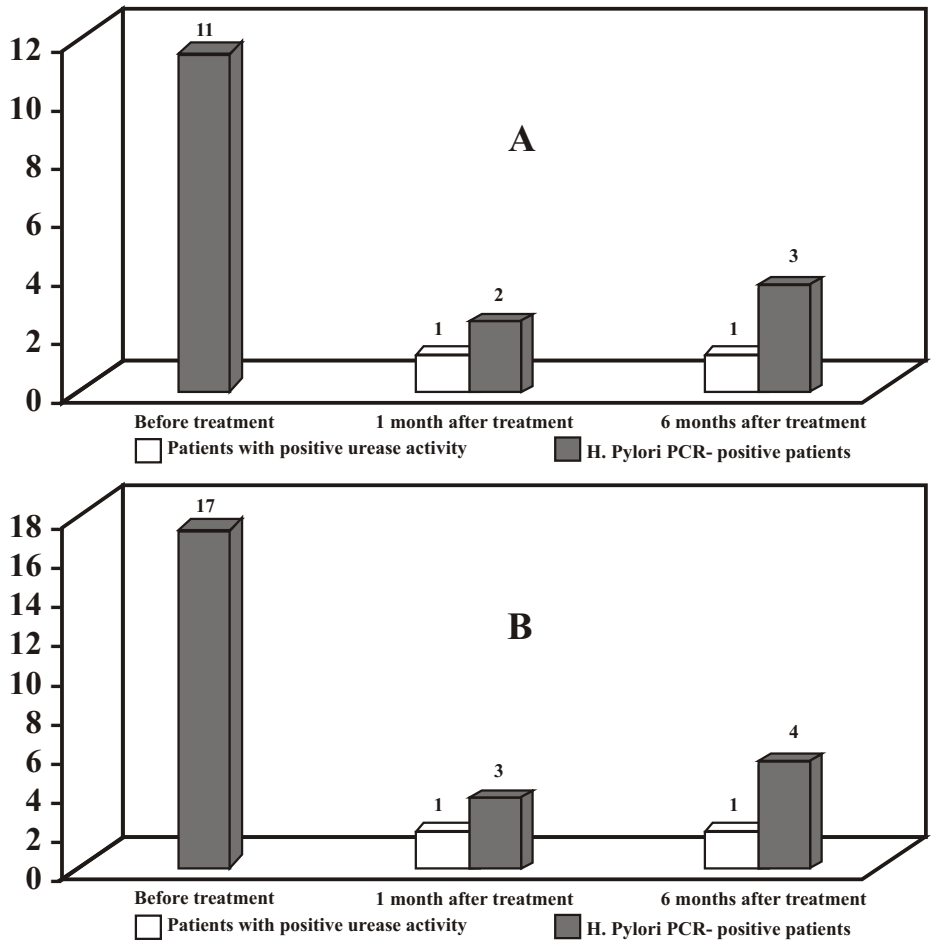

Fig -2

H. Pylori detection in stomach mucosa bioptats with urease activity method and PCR in the examined patients of the first (A) and the second (B) groups before, immediately after andafter 1 and 6 months of anti H. Pylori treatment (see explanations in the text)

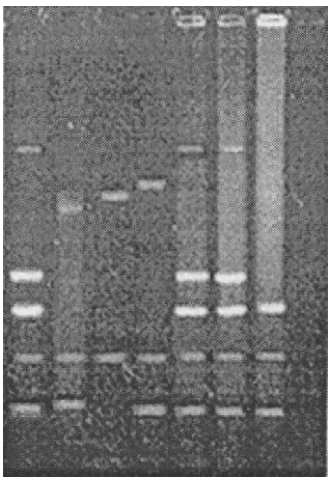

Fig -3

Electrophoregrams of the products restriction of amplified *flaA* fragments *H. Pylori.* Differences of restriction's pattern show the presence of 5 major *H. Pilori.* strains

References

1. Owen RJ. Helicobacter-species classification and identification. Brit. Med. Bul, 1998; 54(1): 17-30.
2. Zh. Ge and D.E. Taylor. Helicobacter pylori-molecular genetics and diagnostic typing. Brit. Med. Bul, 1998;54(1):31-38.
3. Shibata D.K. Polymerase chain reaction and molecular-genetic analysis of bioptats. In: Herrington CS and McGee JOD, Eds. Diagnostic molecular pathology. A practical approach. M: " ", 1999: 395-447.
4. Taylor NS, Fox JG, Akopyants NS, Berg DE, Thompson N, Shames B, Yan L, Fontham E, Janney F, Hunter FM, and Correa P. Long-Term Colonization with Single and Multiple Strains of Helicobacter pylori Assessed by DNA Fingerprinting. J. Clin. Microbiol, 1995; 33 (4): 918-923.
5. UlfT. Molecular diagnosis of Helicobacter pylori. Immunol. Invest., 1997;26(1&2):163-174.
6. Loginov AS, Ilchenko AA, Mukamolova GV, Fedulova NG, Dudik TV, Kaprelyants AS, Reshetnyak VI. Comparative efficiency of different Helicobacter pylori elucidation methods in patients with peptic ulcer. Rus. Gastroenterol. J., I998;3:3-ll
7. Loginov.S, Reshetnyak VI, Mukamolova GV, FedulovaNG, Ilchenko AA, Dudik TV, Kaprelyants AS. Dormant Helicobacter pylori in gastric mucosa in treated patients with ulcer. Ther. Archiv, 1999;2:13-16.
8. Loginov AS, Reshetnyak VI, Kaprelyants AS, Mukamolova GV, Fedulova NG, Dudik TV, Ilchenko AA. The possibility of existence of Helicobacter pylori cells in dormant state in gastric mucosae. Abstracts 11th World Congresses of Gastroenterology. Vienna, Austria. September 6-11, 1998; p. 471.