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An Adaptive Evolution of *Helicobacter pylori*: Role of the *CagA* Presence in the Outcome of *H. pylori* Eradication in Children

Alexander A Nijevitch*, Bulat Idrisov, Ekaterina Kuchina, Fanil Bilalov and Elsa N Akhmadeeva

Bashkortostan State Medical University, Pediatrics Department, 3 Lenina St., Ufa, Russia

Abstract

Introduction: The aim of the study was to determine the evolutionary role of the *CagA* presence in the outcome of eradication treatment of *H. pylori*.

Materials and methods: Sixty-seven pediatric dyspeptic patients (median age 13.7 years, range 5 to 17 years, male/female 24/43) underwent endoscopy for *H.pylori* presence. Gastric biopsy specimens were taken for histology and/or culture and one biopsy sample was used for *CagA* PCR determination. *H. pylori* positive patients were treated for 2 weeks by amoxycilline (50 mg/kg/day), bismuth subcitrate (8 mg/kg/day), nifuratel (30 mg/kg/day) plus omeprazole (1 mg/kg, once daily).

Results: Forty one of 67 children (61.2%) were *H. pylori* positive. Nineteen of 41 strains (46.3%) were *CagA* positive and 22 were *CagA* negative (53.7%). *H. pylori* was eradicated in 33 patients (80.4%). Among the patients with successful eradication 18 children were *CagA* positive, fourteen were *CagA* negative.

Thus, more patients with *CagA*-negative status had evidence of ongoing *H. pylori* infection (36.4% (8/22) versus 5.3% (1/19); χ^2=4.08, p=0.0021; Fisher's exact test p=0.0238).

Conclusion: In our study the carriage of *CagA*-lacking strain was associated with failure of treatment. In our opinion, this phenomenon, related to extraordinary genome plasticity, not only allows the microbe to maintain balanced relationship with the host, but also to survive in antibacterial therapy conditions.

Keywords: Evolution; *Helicobacter pylori*; Children; Eradication; Nifuratel

Introduction

A resistance to antibacterial drugs is a classic demonstration of natural selection in microorganisms during the period of host-bacterial interaction and high intensity of antibiotic exposure [1]. Many bacterial pathogens probably have affected humans for more than 15,000 years [2]. It is obvious, that the host-bacterial interaction can potentially be accompanied with microevolution of bacteria during long-term colonization protracting for lifelong persistence. Furthermore, subclinical infections are more common than apparent disease; bacteria may modulate their virulence in ways that prolong the interactions with the host to optimize transmission [3]. Some of the genes associated with antibacterial resistance have a long evolutionary history of diversification that began well before the antibiotic era [1]. Environmental forces (the release of large amounts of antibacterials *et cetera*) might change natural ecosystem to alteration of the population dynamics of the microorganisms, including selection of resistance with consequences for human health that is difficult to predict [4]. There is an evidence that *Helicobacter pylori* (*H. pylori*) has been colonizing humans for more than ten millions years or longer [2]. *H. pylori* is a gram-negative microaerobic, spiral bacterium that colonizes stomachs of approximately over 50% of the world human population. *H. pylori* infection is associated with chronic gastritis and peptic ulceration development and is also considered to be a risk factor for development of gastric cancer [2]. There are many factors that might be important for treatment outcome, including age *CagA* status, clinical presentations (e.g dyspesia vs. duodenal ulcer) *et cetera* [5,6]. An important virulence determinant of *H. pylori* is a *CagA* gene product which is found in approximately 40% isolates obtained from children [6]. The *CagA* gene is located at one end of the *cag* pathogenicity island (PAI), an approximately 40-kilobase region that is incorporated into the *H. pylori* genome by horizontal transfer from an unknown natural source [7]. Most studies considered *cagA* as a marker of the *cag* PAI [8,9]. The *CagA*-positive strains are associated with a higher grade of gastric or duodenal inflammation and are more virulent than the strains lacking it. Furthermore, the *CagA* gene of *H. pylori* is assumed as responsible for signaling mechanisms that lead to development of gastric adenocarcinoma [10]. Deletion of *cag* PAI by an inductive signal or selective forces may promote the readaptation of the pathogen at different stages of infection [3]. Taking into consideration that *CagA* gene (and *cag* PAI) are the basic virulence factors, the excision of cag PAI may demonstrate that attenuating the virulence of *H. pylori* strain can be an evidence of development of favourable adaptation. The aim of the study was to determine the evolutionary role of the *CagA* presence in the outcome of eradication treatment of *H. pylori*.

Materials and Methods

Between January 2009 and February 2010, 67 pediatric patients with upper dyspepsia (median age 13.7 years, range 5 to 17 years, male/female 24/43) undergoing endoscopy in the Outpatient Department of Children`s Republican Hospital of Bashkortostan Republic (Ufa, Russian Federation) were investigated for *H. pylori* presence. Fully informed parental consent was obtained in all cases. Upper gastrointestinal endoscopy was carried out after lidocaine throat anaesthesia using Olympus XP 20 gastroscope (Japan). Gastric

***Corresponding author:** Alexander A Nijevitch, Bashkortostan State Medical University, Pediatrics Department, 3 Lenina St., Ufa, Russia, 450008; E-mail: aanj@yandex.ru

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antral biopsy specimens were taken for histology (Giemsa staining) and/or culture and one biopsy fragment was used for *CagA* PCR determination. The endoscope and biopsy forceps were disinfected in 2% glutaraldehyde solution after each use. The presence of *H. pylori* infection on histology/culture was taken as diagnostic of the infection [11]. Culture of *H. pylori* was performed on nonselective and selective media (standard Columbia agar with 10% of sheep`s red blood cells) before treatment initiation. The *CagA* status was determined by PCR after DNA extraction. The biopsy samples were ground with tissue homogenizer and resuspended in 300 µl of extraction buffer (20 mM Tris-HCl, pH 8, 0.5% Tween 20), and proteinase K (0.5 mg/ml) was added. The mixture was incubated for 1 hour at 56ºC. Finally, the enzyme was inactivated by boiling for 10 minutes [9]. The *CagA* status was determined by amplification of fragments of the *CagA* gene, as described by Govorun et al. [12]. A positive *CagA* status was defined as positive *CagA* PCR results with one of the two primer sets (e.g Figure 1).

H. pylori positive patients were treated with a two-week course of amoxycilline (50 mg/kg/day), bismuth subcitrate (8 mg/kg/day), nifuratel (30 mg/kg/day) plus omeprazole (1 mg/kg, once daily) [13]. The criterion for eradication of *H. pylori* was the negative breath ammonia test (Helic test, AMA, Saint-Petersburg, Russia) in 4-6 weeks after the treatment completion. Also compliance was estimated in both groups at the end of the treatment and was graded as excellent if over 80% of the provided drugs had been used, fair if 60-80% had been used and poor if less than 60% had been used. Statistical analysis was performed by using the SAS 9.3 statistical package (SAS Institute, 2011) [14]. The significance of difference in categorized data was tested by the chi-squared test and Fisher's exact test. For the both tests, a P-value under 0.05 was considered to have statistical significance. Estimated odds ratios (OR) and 95% confidence intervals (95% CI) were calculated as well.

Results

Forty one of 67 children (61.2%) undergoing endoscopy were *H. pylori* culture positive initially. Nineteen of 41 strains (46.3%) were *CagA* positive (group I) and 22 were *CagA* negative (53.7%) (group II). Four-six weeks after stopping the treatment, *H. pylori* was eradicated in 32 (78.04%) patients overall. Among the patients with successful eradication there were 18 children from *CagA*-positive sample (group I) and 14 from *CagA*-negative sample (group II). Thus, more patients in group II (*CagA*-negative status) had evidence of ongoing *H. pylori* infection (36.4% (8/22) versus 5.3% (1/19); χ^2=4.08, *p*=0.0021; Fisher's exact test *p*=0.0238). Nine children from both groups (21.96%) remained *H. pylori* positive. So, all of the unsuccessfully treated patients were *CagA*-negative initially, with the exemption of one *CagA*-positive carrier. It seems that the carriage of *CagA*-lacking strains of *H. pylori* are associated with unsuccessful eradication according to our data (OR=10.28; 95CI, 1.148 to 92.173). Importantly, compliance was equal in both groups, no patients dropped out the study (over 80% of the provided drugs had been used by all patients).

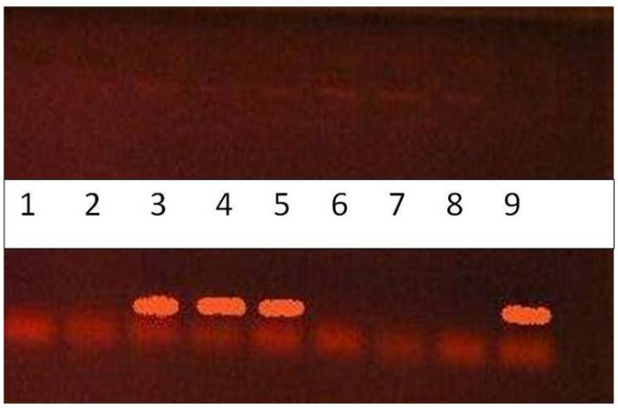

Figure 1: PCR inferred results of *cagA* gene. 2% gel electrophoresis of *H. pylori* genotypes showing PCR results od *cagA* gene. Lane 1 is negative control; lanes 3,4,5 showed PCR products of *cagA* genes; lanes 2,6,7 and 8 are *cagA* negative, lane 9 is positive control.

Discussion

From a biological point of view, the explanation of relationship between *H. pylori* eradication outcome and *CagA* status can have many reasons:

1. *CagA*-positive strains grow faster and would be more susceptible to antibiotics with the interfering metabolism dividing cells than *CagA*-negative ones, which may be in stationary phase;

2. Strain, expressing *CagA*, enhance production of proinflammatory cytokines and increase inflammation in gastric mucosa (and mucosal blood flow respectively) which may be favourable for antibiotics diffusion;

3. Proinflammatory cytokines are potent inhibitors of gastric acid secretion and can potentiate efficacy of PPI action [9,15].

Earlier, we found inverse negative relationship between *CagA* carriage and *rdxA* gene deletion in *H. pylori* strains (p=0.0001) [16]. Also it is well known that, alterations in the *rdxA* gene of *H. pylori* are required for moderate metronidazole resistance [17]. Similar data was presented by other authors as well [18]. Therefore, absence of *cag* PAI can be a risk factor for metronidazole resistance and plays a crucial role in successful eradication of *H. pylori* [19]. However, since we did not include metronidazole to eradication regimen in our particular study its resistance role is unconsidered. Results of the present study can be considered as an illustration of successful adaption of genome of *H. pylori* to environmental influences (antibiotics, host physiology). From our point of view, the evolutionary forces are capable to demonstrate a strong influence on the variable region of genes of virulence (*CagA* in this case) with low biological cost to the host and the microbe. The strains of *H. pylori* lacking the *cag* PAI, or parts of it, have lost the island through transformation with an empty site or through deletion mutations [20]. It is possible that this particular phenomenon associated with genome plasticity not just allows the microbe to maintain balanced relationship with the host, but also to survive in antibacterial therapy conditions.

The obtained results seem to reveal the source of genetic diversity among the strains and differences in risk for outcome of gastroduodenal pathology among human carriers of *H. pylori* [21]. Possibly, successful adaption to evolutionary forces also contributes to extraordinary chronicity of *H. pylori* infection [22]. Natural selection which helps to fix particular mutants or recombinant genotypes with beneficial alleles could turn a host-pathogen interaction into almost symbiotic relationship. As it was shown by P. Ewald, for agents of disease that are spread directly from person to person, low virulence is more preferable, as it allows the host to remain active and contact with other potential hosts [23]. An organism that kills his host (lethal outcome of peptic ulcer or gastric cancer) may never obtain a new host, so natural selection would seem to favour lower virulence. The colonies of *H. pylori* with functional cag PAI could be potentially more harmful to

humans than those lacking it, at least due to the fact that gastric cancer is a highly lethal disease.

Undoubtedly, the study of virulence determinants of *H. pylori* that have been subject to natural selection in particular populations can provide new understanding of mechanisms in effective eradication of this infectious agent in pediatric and adult patients worldwide.

Unfortunately, our study has several important limitations. Further research on the evolutionary role of the *CagA* presence in the outcome of eradication treatment of *H. pylori* is needed with larger sample sizes. To generalize the results, we should have included more participants at different levels since our study involved relatively low number of patients. Also it is obvious that, the PCR on *cagA* gene is an indirect evidence of a presence or absence of the pathogenicity island and the *post hoc* fallacy might have been remained since the loss of *cagA* gene is not only one risk indicator of the unfavourable outcome. However, strength for interpretation of the current results is derived from the fact that our research is the only one study in the given field from the middle part of Russia so far. Our study provides supplemental information to help resolve the worldwide conundrum over the evolutionary role of the *CagA* presence in the outcome of *H. pylori*-associated disease. Despite all the limitations in our work, we regard further investigations of evolutionary role of *CagA* carriage are necessary.

Conclusion

Our data again confirmed the opinion of a number of researchers on higher survivability of *H. pylori* European strains, not carrying functional *CagA* in intensive antibacterial therapy conditions [9,10]. Further studies are needed to assess the role of the evolutionary process in which bacteria are losing their virulence factors for better survivability in symbiosis with humans.

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