Original Article

The effect of virulence genotypes of *Helicobacter pylori* on eradication therapy in children

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Abstract

Background/Aim: It is important to eradicate *Helicobacter pylori* at an early stage in patients during childhood to potentially prevent the development of *H. pylori*-related diseases. Studies have demonstrated that the virulence genotype of *H. pylori* influences the efficacy of eradication therapy. The efficacy of triple therapy has decreased significantly, which has seriously affected the clinical outcome of children with *H. pylori* infection. In this study we aimed to investigate the influence of virulence genotypes of *H. pylori* on triple eradication therapy in children.

Patients and Methods: *H. pylori* strains were isolated from the gastric antrum mucosa in children with upper gastrointestinal symptoms. Polymerase chain reaction (PCR) was conducted to determine the *H. pylori cagA*, *vacA*, and *iceA* genotypes. All patients with *H. pylori* infection were administered 14-day triple therapy. After drug withdrawal for at least 4 weeks, the ^{13}C-urea breath test (^{13}C-UBT) was used to observe the therapeutic effect of *H. pylori* eradication. The eradication rates were evaluated by intention-to-treat (ITT) and per-protocol (PP) analyses.

Results: A total of 107 patients were enrolled in this study. Nine patients were lost to follow-up, and 98 patients were administered eradication therapy. Based on ITT and PP analyses, the *H. pylori* eradication rate was 64.5% (69/107) and 70.4% (69/98), respectively. Among the successful eradication groups, the *cagA*-positive, *vacA*s1a, *vacA*s1c, *vacA*m1, *vacA*m2, *iceA*1, and *iceA*2 genes were identified in 72.8%, 68.1%, 76.9%, 60.0%, 74.6%, 71.8%, and 75.0% of strains, respectively. Of the unsuccessful eradication groups, the *cagA*-positive, *vacA*s1a, *vacA*s1c, *vacA*m1, *vacA*m2, *iceA*1, and *iceA*2 genes were identified in 27.2%, 31.9%, 23.1%, 40.0%, 25.4%, 28.2%, and 25.0% of strains, respectively. No statistically significant differences were noted in the detection rate of the *H. pylori* genotypes between the *H. pylori* successful and unsuccessful eradication groups ($P > 0.05$).

Conclusions: The *cagA*, *vacA*, and *iceA* genotypes of *H. pylori* are not associated with the efficacy of omeprazole-based triple therapy on the eradication of *H. pylori* infection in children.

Keywords: Child, *Helicobacter pylori*, polymerase chain reaction, triple therapy, virulence genotype

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is associated with various diseases of the upper gastrointestinal tract, such as gastritis, peptic ulcers, and mucosa-associated lymphoid tissue in children.[1] It is important to eradicate *H. pylori* at an early stage in patients during childhood to potentially prevent the development of *H. pylori*-related diseases. The main virulence genes of *H. pylori* include *cagA*, *vacA*, and *iceA*. At present, the recommended therapy for eradicating *H. pylori* in pediatric patients is triple therapy, which consists of a proton pump inhibitor (PPI) and two other antibiotics.[2] The efficacy of triple therapy has decreased in children. The reasons for failure in eradication mainly include *H. pylori* strain factors, host factors, environmental factors, inappropriate treatment, and low compliance to therapy. This study aimed to investigate the influence of *H. pylori* virulence genotypes on 14-day omeprazole-based triple therapy with two antibiotics by detecting the *cagA*, *vacA*, and *iceA* status of *H. pylori* in children.

PATIENTS AND METHODS

From July 2014 to September 2015, 107 patients who suffered from abdominal pain, vomiting, belching, and gastrointestinal bleeding and who underwent gastroscopy were enrolled in this study. After informed consent was obtained, gastric biopsies were collected by a clinical gastroenterologist. The exclusion criteria were as follows: (1) patients who had taken any PPI, H_2 receptor antagonists, bismuth salts, or antibiotics for at least 4 weeks prior to the time of their enrolment in the study; (2) patients whose condition was complicated by severe heart, lung, blood, liver, or kidney dysfunction; and (3) patients who would not comply to the study. The ethics committee of the Children's Hospital of Jiangxi approved the study, NO. JXSETYY-2016003. Informed consent was obtained from the parents or guardians of the children enrolled in the study.

H. pylori isolation and culture

Gastric mucosal biopsy specimens were used for *H. pylori* culture. The biopsies from each patient were cultured on the surface of Karmali agar (Oxoid, Basingstoke, Hampshire, England) plates. The cultured plates were incubated in microaerobic atmosphere conditions (37°C, 5% oxygen, 10% carbon dioxide, and 85% nitrogen) for 3 to 7 days. After culture, smooth, neat, gray, circular, translucent colonies approximately 0.5 to 2 mm in diameter were identified as *H. pylori* based on a rapid urease test and modified Giemsa staining; consistent positive results between the two tests was required. The bacterial isolates were preserved in brain heart infusion broth enriched with 20% glycerol and 10% inactivated horse serum and were stored at −80°C. The standard NCTC11639 *H. pylori* strain was donated by the Institute of Digestive Diseases, the First Affiliated Hospital of Nanchang University.

H. pylori eradication therapy

Patients received 14-day triple therapy consisting of omeprazole (0.6–0.8 mg/kg, bid), amoxicillin (50 mg/kg/day, bid), clarithromycin (15–20 mg/kg/day, bid), or metronidazole (20 mg/kg/day, bid). For patients who were allergic to penicillin, amoxicillin was replaced with metronidazole. For patients with gastrointestinal bleeding and peptic ulcers, omeprazole (0.6–0.8 mg/kg, qd) was administered for an additional 2–4 weeks after the 14-day treatment.

PCR amplification of *H. pylori* virulence genotypes

Genomic DNA was extracted from *H. pylori* strains isolated from gastric biopsy specimens using the QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was stored at −20°C until it was used for polymerase chain reaction (PCR) amplification. PCR was performed on purified genomic DNA from all *H. pylori* isolates to examine the presence of virulence genotypes, including *cagA*, *vacA*, and *iceA*. Primers were designed based on published papers and are listed in Table 1.[3-5] The primer sequences for *H. pylori* DNA were synthesized by GenScript Co. Ltd. (Nanjing, China). Each PCR was performed in a total volume of 25 μl. The final reaction mixture contained 500 ng of genomic DNA, 8.5 μl of distilled water, and 12.5 μl of 2 × Taq PCR master mix (TIANGEN, KT201, Beijing, China), including 0.1 U/μl Taq Polymerase, 500 μmol/L of each dNTP, 20 mmol/L Tris-HCl (pH 8.3), 100 mmol/L KCl, 3 mmol/L $MgCl_2$, and 2 μl of each primer (forward and reverse mixture). The PCR amplification conditions were as follows: pre-denaturation was performed at 95°C for 5 min; 30 cycles of 30 s at 94°C, 30 s at 55°C (for *ureA*, *cagA*, *vacA*s1, *vacA*s1a, *vacA*s1b, *vacA*m1, *vacA*m2, *iceA1*, and *iceA2*) or 59°C (for *vacA*s1c and *vacA*s2) for annealing; and a final extension at 72°C for 7 min. The final reactions were stored at 4°C. PCR products were electrophoresed on a 2.0% (w/v) agarose gel (Sigma, USA) that was stained with 0.5 μg/ml ethidium bromide and developed under UV light (Bio-Rad, USA) according to the standard procedures. A 100-bp DNA ladder (TIANGEN, D2000, Beijing, China) served as a molecular size marker.

Eradication assessment

At least 4 weeks after the completion of treatment, bacterial eradication was assessed using a ^{13}C-urea breath

test (^{13}C-UBT). ^{13}C-UBT employed a ^{13}C-urea 75-mg capsule (Zhonghe Headway Bio-sci and Tech Co, Ltd, Shenzhen, China). When the 30-min Delta over baseline value was 4 or more, the patient was determined to be positive for *H. pylori*. A negative result was regarded as an eradication success, whereas a positive result was regarded as an eradication failure.[2]

Statistical analysis

Statistical analysis was performed using (SPSS Inc., Chicago, IL, USA). The eradication rates were evaluated by intention-to-treat (ITT) and per-protocol (PP) analyses. Chi-square test was used for comparisons between the groups. A *P* value of <0.05 was considered statistically significant.

RESULTS

Characteristics of the study group

Of all the 107 children, 98 completed eradication therapy and received ^{13}C-UBT after treatment. Among these patients, 77 were males and 21 were female. The patients ranged from 4 to 14 years old. According to the endoscopy diagnoses, 29 patients had chronic gastritis, 12 had duodenal bulb inflammation, and 57 had peptic ulcers (3 had gastric ulcers and 54 had duodenal ulcers). Nine patients did not complete the treatment. Among these patients, 2 were too young to receive eradication therapy, 1 patient discontinued treatment due to a rash, 4 patients did not receive ^{13}C-UBT after treatment, and 2 patients were lost to follow-up.

Eradication of *H. pylori*

Among 98 patients, 69 had a negative result for ^{13}C-UBT and were regarded as eradication success. The eradication rates according to ITT and PP analysis were 64.5% (69/107) and 70.4% (69/98), respectively.

Analysis of *cagA*, *vacA*, and *iceA* genotypes

Among 98 strains, the *cagA* subtypes were detected in 92 strains of *H. pylori* isolates. The number of strains positive for *vacA*s1a, *vacA*s1c, *vacA*m1, and *vacA*m2 subtypes were 72, 26, 30, and 67 strains, respectively. One strain was both *vacA*m1 and *vacA*m2 positive. The number of strains positive for *vacA*s1a/m1, *vacA*s1a/m2, *vacA*s1c/m2, and *vacA*s1c/m1 subtypes was 26, 49, 4, and 18 strains, respectively. However, the *vacA*s1b and *vacA*s2 subtypes were not detected in any samples. In addition, the number of strains positive for *iceA*1 and *iceA*2 subtypes was 78 and 8 strains, respectively, whereas 8 strains were both *iceA*1 and *iceA*2 positive [Table 2].

Association of *H. pylori* genotypes with eradication rates

Among the successful eradication groups, the *cagA*, *vacA*s1a, *vacA*s1c, *vacA*m1, *vacA*m2, *iceA*1, and *iceA*2 genes were identified in 72.8%, 68.1%, 76.9%, 60.0%, 74.6%, 71.8%, and 75.0% of strains, respectively. Of the unsuccessful eradication groups, the *cagA*-positive, *vacA*s1a, *vacA*s1c, *vacA*m1, *vacA*m2, *iceA*1, and *iceA*2 genes were identified in 27.2%, 31.9%, 23.1%, 40.0%, 25.4%, 28.2%, and 25.0% of strains, respectively. No statistically significant differences in the prevalence of *H. pylori* genotypes were noted between the successful and unsuccessful eradication groups ($P > 0.05$) [Table 3].

Table 1: PCR primers for the amplification of *H. pylori cagA*, *vacA* and *iceA* sequences

Amplified region	Primer sequence (5'→3')	PCR product (bp)	Reference
cagA	5'-GATAACAGGCAAGCTTTTGAGG-3 5'-CTGCAAAAGATTGTTTGGCAGA-3	349	4
*vacA*s1	5'-ATGGAAATACAACAAACACAC-3' 5'-CTGCTTGAATGCGCCAAAC-3'	259	4
*vacA*s2	5'-GCTTAACACGCCAAATGATCC-3' 5'-CTGCTTGAATGCGCCAAAC-3'	199	5
*vacA*s1a	5'-GTCAGCATCACACCGCAAC-3' 5'-CTGCTTGAATGCGCCAAAC-3'	190	3
*vacA*s1b	5'-AGCGCCATACCGCAAGAG-3' 5'-CTGCTTGAATGCGCCAAAC-3'	187	3
*vacA*s1c	5'-CTCGCTTTAGTGGGGCTA-3' 5'-CTGCTTGAATGCGCCAAAC-3'	213	3
*vacA*m1	5'-CAATCTGTCCAATCAAGCGAG-3' 5'-GCGTCTAAATAATTCCAAGG-3	570	4
*vacA*m2	5'-GGAGCCCCAGGAAACATTG-3' 5'-CATAACTAGCGCCTTGCAC-3'	352	3
iceA1	5'-GTGTTTTTAACCAAAGTATC-3' 5'-CTATAGCCACTTTCTTTGCA-3	247	4
iceA2	5'-GTTGGGTATATCACAATTTAT-3' 5'-TTACCCTATTTTCTAGTAGGT-3	229/334	4

Table 2: Prevalence of *H. pylori* genotypes detected in strains

Genotype	Total number (*n*)	Positive number (*n*)	Detection rate (%)
cagA-positive	98	92	93.9
*vacA*s1	98	98	100.0
*vacA*s2	98	0	0
*vacA*s1a	98	72	73.5
*vacA*s1b	98	0	0
*vacA*s1c	98	26	26.5
*vacA*m1	98	30	30.6
*vacA*m2	98	67	68.4
*vacA*m1/*vacA*m2	98	1	1.0
*vacA*s1a/m1	98	26	26.5
*vacA*s1a/m2	98	49	50.0
*vacA*s1c/m1	98	4	4.1
*vacA*s1c/m2	98	18	18.4
*iceA*1	98	78	79.6
*iceA*2	98	8	8.2
*iceA*1-positive/*iceA*2-positive	98	8	8.2

DISCUSSION

H. pylori is a Gram-negative, spiral, microaerophilic bacterium. The bacteria can adhere to the surface of the gastric mucosal epithelium and the bottom layer of the

Table 3: Relationship between the detection rate of *H. pylori* genotypes and treatment outcome

Genotype	Total number (*n*)	Successful Eradication (%)	Unsuccessful Eradication (%)	χ^2	*P*
cagA-positive	92	67 (72.8)	25 (27.2)	2.534	0.111
cagA-negative	6	2 (33.3)	4 (66.7)		
*vacA*s1a	72	49 (68.1)	23 (31.9)	0.721	0.396
*vacA*s1c	26	20 (76.9)	6 (23.1)		
*vacA*m1	30	18 (60.0)	12 (40.0)	2.115	0.146
*vacA*m2	67	50 (74.6)	17 (25.4)		
*vacA*s1a/m1	26	15 (57.7)	11 (42.3)	2.679	0.444
*vacA*s1a/m2	49	37 (75.5)	12 (24.5)		
*vacA*s1c/m1	4	3 (75.0)	1 (25.0)		
*vacA*s1c/m2	18	13 (72.2)	5 (27.8)		
*iceA*1	78	56 (71.8)	22 (28.2)	0.365	0.833
*iceA*2	8	6 (75.0)	2 (25.0)		
*iceA*1-positive/*iceA*2-positive	8	5 (62.5)	3 (37.5)		

gastric mucosa, and the infection status can be lifelong if the infected person does not undergo standardized treatment.[6] At present, the recommended therapy to eradicate *H. pylori* in children involves triple therapy that consists of a PPI and two additional antibiotics.[2] In regard to eradication therapy, PPIs have an anti-*H. pylori* effect. The bactericidal effect of antibiotics depends on strong acid inhibition of PPI, whereas PPIs and antibiotics have synergistic bactericidal effects. Thus, PPI-based triple therapy may increase the efficacy of *H. pylori* eradication therapies.[7] Given the development of this triple therapy and continued widespread use of antibiotics in general practice in recent years, the failure rate of this triple therapy has increased among children. *H. pylori* eradication treatment may fail for a number of reasons, including antibiotic resistance, bacterial virulence factors, environmental factors, different diseases, and low compliance to therapy.[8-12] *H. pylori* virulence genes include genes such as *vacA*, *cagA*, and *iceA*. The cytotoxin-associated gene A (*cagA*) gene, which encodes cytotoxin-associated A protein (CagA), enters host epithelial cells of the stomach using a type IV secretion system, undergoes phosphorylation, interferes with host cell signalling pathways, and produces severe inflammation and tissue damage. Vacuolating cytotoxin gene A (*vacA*) encodes vacuolating cytotoxin A (VacA). The *vacA* genetic structure has alleles of the mosaic structure, including the signal (s) and middle (m) regions. The "s" and "m" regions are divided into s1 (s1a, s1b, and s1c) or s2 and m1 or m2 subtypes, respectively. The induced by contact with epithelium (*iceA*) gene has two main allelic variants – *iceA1* and *iceA2*. Several studies have reported that the virulence genotype of *H. pylori* may play an important role in the development of gastrointestinal disease.[13,14]

This study focused on 107 children who were positive for *H. pylori* culture and were cured with a 14-day triple therapy with omeprazole, amoxicillin, clarithromycin, or metronidazole. In total, 98 patients completed the treatment, and 69 exhibited successful eradication based on ^{13}C-UBT after treatment. The eradication rate according to ITT and PP analyses were 64.5% (69/107) and 70.4% (69/98), respectively. Over the past decades, standard triple therapy contributed to a successful eradication rate of greater than 90% in children. However, successful eradication rates have significantly declined. The emergence of *H. pylori*-resistant strains is one of the most important causes of the reduction in the eradication rate of triple regimens with a PPI plus two antibiotics. At present, the most common resistance to antibiotics worldwide among affected children involve metronidazole and clarithromycin, whereas the resistance rate of amoxicillin is lower.[15,16] In China, the rates of resistance to metronidazole and clarithromycin were increased among children infected with *H. pylori* with resistance rates of 49.2% and 34.9%, respectively. The amoxicillin resistance rate was 6.2%.[17] In Japan, the rates of resistance to metronidazole and clarithromycin were 43.3% and 21.9%, respectively, among children infected with *H. pylori*.[18] In Turkey, the rates of resistance to metronidazole and clarithromycin among children infected with *H. pylori* were 48.4% and 30.1%, respectively; resistance to amoxicillin was not observed.[19]

The *cagA*, *vacA*, and *iceA* genes are the major virulence genes of *H. pylori*. Studies have assessed the relationship between *H. pylori* genes and eradication therapy. The results of this study demonstrated that, among the groups that underwent successful eradication, the *cagA*, *vacA*s1a, *vacA*s1c, *vacA*m1, *vacA*m2, *iceA*1, and *iceA*2 genes were identified in 72.8%, 68.1%, 76.9%, 60.0%, 74.6%, 71.8%, and 75.0% of strains, respectively. Of the groups that did not undergo successful eradication, the *cagA*, *vacA*s1a, *vacAs1c*, *vacA*m1, *vacA*m2, *iceA*1, and *iceA*2 genes were identified in 27.2%, 31.9%, 23.1%, 40.0%, 25.4%, 28.2%, and 25.0% of strains, respectively. No statistically significant differences in the detection rate of *H. pylori* genotypes were noted between the successful and unsuccessful *H. pylori* eradication groups ($P > 0.05$). These results suggest that

the *cagA*, *vacA,* and *iceA* genotypes of *H. pylori* may have no relation to the efficacy of omeprazole-based 14-day triple therapy in children. A study published by Van Doorn *et al.*[20] suggested that the eradication rates of *H. pylori* were increased for *cagA*-positive and *vacA*s1 *H pylori* strains, a finding that is consistent with the increased eradication rate observed among ulcer patients compared with functional dyspepsia patients. Khan *et al.*[21] demonstrated that the alarming levels of antibiotic resistance were associated with the *cagA* gene in *H. pylori* strains. The results of this study are inconsistent with previous reports, which may be related to the differences in *H. pylori* infection rates between children and adults, and the different regions and *H. pylori* strain polymorphisms in different regions. The *cagA*-negative and *vacA*s2 *H. pylori* strain may increase the risk of *H. pylori* eradication failure, suggesting that *cagA* and *vacA* genotypes play an important role in *H. pylori* eradication therapy. A possible reason is that *cagA*-positive strains can induce severe inflammatory reactions in the gastric mucosa. When gastric mucosal inflammation is obvious, the blood flow in the mucosa is rich, which is beneficial to the efficacy of antibiotics in the gastric mucosa compared with the *cagA*-negative strains. In the gastric mucosa, IL-1β can inhibit gastric acid secretion. The *cagA*-positive strain noted in children produces significantly increased IL-1β in the gastric mucosa compared with *cagA*-negative strains, which produce less gastric acid secretion. This feature makes the area more accessible to antibiotics and conducive to curing *H. pylori.* The *vacA*s1 and *vacA*m1 genotypes of *H. pylori* have been associated with severe gastric inflammation, produce a large amount of toxins, and induce increased vacuolating activity in gastric epithelial cells. Thus, this genotype enhances the antibacterial effect of antibiotics, whereas the *vacA*s2 and *vacA*m2 genotypes produce minimal or no toxins.[22,23]

In conclusion, in areas of low (<20%) clarithromycin resistance, triple therapy involving PPI and two antibiotics should be recommended as first-line treatment for children with *H. pylori* infection. The results of this study indicate that the eradication rate of *H. pylori* in children has declined considerably compared with previous studies. In addition, the *cagA*, *vacA,* and *iceA* genotypes of *H. pylori* are not associated with the efficacy of 14-day omeprazole-based triple therapy on the eradication of *H. pylori* infection in children. Since the sample size in this study is limited, a multicenter, randomized controlled trial with a large sample size is needed to further address the issue.

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Conflicts of interest

There are no conflicts of interest.

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