Presence of gastric Helicobacter species in children suffering from gastric disorders in Southern Turkey

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Abstract

Background: Infections with gastric *Helicobacter* spp. are associated with gastritis, peptic ulceration and malignancies. *Helicobacter pylori* is the most prevalent *Helicobacter* species colonizing the human stomach. Other gastric, non-*H. pylori* helicobacters (NHPH) have been described in 0.2-6% of human patients with gastric disorders. Nevertheless, due to difficulties in the diagnosis of NHPH infections and lack of routine screening, this is most likely an underestimation of their true prevalence. To the best of our knowledge, no studies have been performed on the presence of *Helicobacter* spp. in children suffering from gastric disorders in Southern Turkey.

Materials and methods: In total, 110 children with gastric complains were examined at the Cukurova University Balcali hospital, Turkey. Gastroscopy was performed to evaluate presence of gastric mucosal lesions. Biopsies of the pyloric gland zone were taken for histopathological analysis, rapid urease testing and presence of *Helicobacter* spp. DNA by PCR.

Results: Based on the PCR results, the prevalence of *Helicobacter* spp. was 32.7% (36/110). *H. pylori* was found in 30.9% (34/110), *H. suis* in 1.8% (2/110) and *H. heilmannii/H. ailurogastricus* in 0.9% (1/110) of the human patients. A mixed infection with *H. pylori* and *H. suis* was present in one patient. The presence of mucosal abnormalities, such as nodular inflammation, ulceration and hyperemia, as well as gastritis was significantly higher in *Helicobacter* spp. positive patients.

Conclusion: *H. pylori*, *H. suis* and *H. heilmannii/H. ailurogastricus* were present in children with gastric complains. Infection with these pathogens may be involved in development of gastritis and ulceration.

Key words: *Helicobacter pylori*, non-*Helicobacter pylori* helicobacters, children, gastric disorders, Turkey

Introduction

Helicobacters are Gram-negative, motile bacteria colonizing the gastro-intestinal tract of humans and animals. *H. pylori* is the best studied and most prevalent *Helicobacter* species colonizing the human stomach. Infections with *H. pylori* are acquired during early childhood and the disease progresses with age [1]. Despite the presence of chronic active gastritis, the majority of *H. pylori* infections remain asymptomatic. Only 10-20% of the *H. pylori* infected people will develop gastric disorders, such as peptic ulcers, mucosa associated lymphoid tissue (MALT)-lymphoma and/or adenocarcinoma [1].

In developing countries, more than 80% of the human population is *H. pylori* positive, while the prevalence rate remains under 40% in developed countries [2]. As the prevalence is rapidly declining in the industrialized world, this may create a niche for colonization of the human stomach by other organisms [3].

Indeed, non-*H. pylori* helicobacters (NHPH) have been demonstrated in 0.2-6% of human patients with gastric complaints. NHPH infections have been associated with development of chronic gastritis, peptic ulcers and MALT-lymphoma. The risk for developing MALT-lymphoma is higher during NHPH infection compared to *H. pylori* infection [4], although the induced gastritis may be less severe [5]. So far, detected NHPH in the human stomach are *H. suis*, *H. felis, H. bizzozeronii, H. salomonis* and *H. heilmannii*. *H. suis* naturally colonizes the stomach of pigs and non-human primates, while the other zoonotic gastric NHPH are mainly associated with dogs and cats [4].

Clinical signs associated with gastric *Helicobacter* spp. infections are acute or chronic epigastric pain and nausea. Other disease signs may include hematemesis, recurrent dyspepsia, irregular defecation frequency and variable stool consistency, vomiting, heartburn, dysphagia and decreased appetite. Gastroscopy may reveal a variety of lesions, ranging from a normal to slightly hyperemic mucosa to mucosal edema and to multiple erosions and ulcerations in the pyloric gland zone and/or duodenum [4].

Various invasive and non-invasive tests have been developed for diagnosis of *H. pylori* infections [6]. In patients who have undergone gastroscopy, these may include rapid urease testing (RUT), cultivation of *H. pylori*, visualization of its curve-shaped morphology at histopathological examination and detection of its DNA in gastric biopsies. Conversely, commercial non-invasive tests are not available for diagnosis of NHPH infections and gastric biopsy samples are not checked routinely for the presence of these bacteria, resulting in a potential underestimation of their true prevalence [7].

The main objective of this study was to obtain better insights in the prevalence of *H. pylori* and NHPH infections in children suffering from gastric disorders in the Southern part of Turkey. Gastroscopy was performed to determine presence of mucosal lesions and biopsies were taken for subsequent histopathological analysis and detection of *Helicobacter* spp. DNA.

Materials and methods

Sample collection

From 2013 to 2014, patients between two and 18-years of age and suffering from gastric disorders were examined at the Balcali hospital, Adana, Turkey. Gastroscopy was performed to analyze presence of mucosal lesions such as hyperemia, nodular inflammation and ulceration.

Using autoclaved forceps, biopsies of 40–50 mg consisting of mucosa and submucosa were taken from the pyloric gland zone for rapid urease testing (RUT), histopathological analysis and DNA extraction. This study was approved by the Ethics Committee of Çukurova University Faculty of Medicine.

RUT and histopathological analysis

RUT was performed using the CLOtest™ (Delta West Ltd., Bentley, Australia) according to the manufacturer's instructions.

The biopsies for histopathological analysis were fixed in 10% phosphate-buffered formalin. Thereafter, they were embedded in paraffin, sectioned at 5 μm, rehydrated, deparaffinized, stained with haematoxylin and eosin (HE), dehydrated and finally mounted with a coverslip for light microscopic evaluation. The severity of gastritis was scored according to the Updated Sydney System [8]. In addition, the stained gastric biopsies were investigated for presence of curve-shaped *H. pylori* bacteria and/or spiral-shaped NHPH bacteria.

Presence of Helicobacter *spp.*

DNA was extracted from gastric biopsies using QIAamp DNA mini kit® (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. These DNA extractions were subjected to a PCR to detect the presence of *H. pylori* and NHPH (i.e. *H. suis, H. heilmannii/H. ailurogastricus, H. felis, H. bizzozeronii* and *H. salomonis*). For *H. pylori*, part of the *ureC* gene was amplified as previously described [9]. For NHPH, part of the *ureA* gene was amplified using genus- and species-specific primers (Table 1). The thermal cycle program consisted of 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 20 s, annealing/extension at 60°C for 30 s and elongation at 72°C for 30 s. The PCR products were analyzed by gel electrophoresis as

104 described elsewhere [10]. To confirm the presence of gastric NHPH DNA, all samples positive
105 for NHPH were sequenced [11].

106 For *H. suis* positive samples, multilocus sequencing typing (MLST) was performed [12,13].

107 *Statistical analysis*

108 Statistical analysis was performed using SPSS statistics 24® (IBM, New York, USA).
109 Differences in macroscopic lesions and severity of gastritis between *Helicobacter* spp. positive
110 and negative patients were investigated using the non-parametric Kruskal-Wallis test with
111 Bonferroni correction for multiple comparisons. Differences were considered statistically
112 significant at a corrected p-value of less than 0.05.

113 **Results**

114 In total, 110 patients, 32 boys and 78 girls, were examined.

115 Based on the results of PCR, the prevalence of *Helicobacter* spp. was 32.7% (36/110). *H. pylori*
116 was found in 30.9% (34/110), *H. suis* in 1.8% (2/110) and *H. heilmannii/H. ailurogastricus* in
117 0.9% (1/110) of the patients. A mixed infection with *H. pylori* and *H. suis* was present in one
118 patient. MLST analysis showed that the strains of both *H. suis* positive patients belonged to the
119 porcine associated sequence types 1 and 4 [12,13].

120 Compared to PCR, RUT showed a sensitivity of 63.89% and specificity of 97.30% to detect
121 presence of *H. pylori* infection. RUT analysis was positive for all three patients infected with
122 gastric NHPH.

123 Detection of curve-shaped bacteria by histopathological analysis showed a sensitivity of 75%
124 and specificity of 95.95%. In none of the samples, bacteria with a long, spiral-shaped
125 morphology were detected.

126 The presence of mucosal lesions as observed at gastroscopy (i.e. hyperemia, nodular
127 inflammation and ulceration), as well as the presence and severity of gastritis as scored at
128 histopathological examination, was significantly higher in *Helicobacter* spp. positive patients
129 ($P<0.01$) (Table 2). No effect of gender could be demonstrated.

130 Although a marked, active chronic gastritis was present in the *H. suis* infected patient, no
131 macroscopic lesions were detected during gastroscopy. In the *H. heilmannii/ailurogastricus*
132 infected patient, hyperemia was observed, but without presence of gastric inflammation. Gastric
133 ulceration in combination with moderate, active chronic gastritis was present in the patient
134 simultaneously infected with *H. pylori* and *H. suis*. Ulcers were also present in three patients
135 only infected with *H. pylori*, but not in the two patients infected with NHPH solely.

136

Discussion

Half of the human population is infected with *H. pylori*, although its prevalence may greatly vary depending on geographical region. Turkey is considered to be an interesting region as it may receive influences from Asian and Western countries showing high and low *H. pylori* prevalence rates, respectively [14]. In Eastern Turkey, *H. pylori* has been detected in 23.6% up to 75.8% of the children [15–17]. For the first time, a prevalence rate of 30.9% was demonstrated in children from Southern Turkey. Compared to Europe (18%), Turkish children are more frequently infected with *H. pylori*, while similar prevalence rates have been detected in Asia (35%) [2]. As described by Yilmaz *et al*., no significant differences in *H. pylori* presence were found between boys and girls [18].

Infection with *H. pylori* has been associated with development of gastritis, peptic ulceration and malignancies [4]. In line with the findings of Uğraş and Pehlivanoğlu, the majority of the *H. pylori* infected children showed presence of gastritis, while only 8% suffered from gastric ulceration [16]. In *H. pylori* infected adults, presence of peptic ulceration may reach 30% [19], indicating that the disease progresses with age [1].

The development of gastric pathologies seems to depend on the present *H. pylori* genotypes. In Western countries and Turkey, *H. pylori* strains predominantly possess cagA type 2a, vacA s1a/m1a and/or vacA m2a genotypes [14], which are linked with gastritis and ulceration [1] [20]. It might be possible that such genotypes were present in the children investigated in this study and which might have contributed to development of gastric pathologies. Nevertheless, this hypothesis needs to be further investigated.

For the first time, gastric NHPH were shown to be present in 2.7% children suffering from gastric disorders in Southern Turkey. This is in line with the described prevalence rates of 0.1-

6% in Europe, Canada, Pakistan, Japan and Thailand [21–29]. In China, higher prevalence rates of up to 12% have been described [30], suggesting that the infection rate of NHPH may differ depending on the geographic region and/or socioeconomic status of the patients.

So far, 5 gastric NHPH species have been described in human patients suffering from gastric disorders. In Belgium, Germany and China it has been shown that *H. suis* is the most common NHPH species in human patients suffering from gastric disorders, followed by *H. salomonis, H. felis, H. heilmannii and H. bizzozzeronii* [9,30]. Similarly, in this study, *H. suis* was most frequently detected. The zoonotic potential of other gastric NHPH, such as *H. cynogastricus, H. baculiformis, H. ailurogastricus, H. acinonychis, H. cetorum* and *H. mustelae,* is currently unknown. The PCR test used in this study is not able to differentiate between *H. heilmannii* and *H. ailurogastricus* as these species possess identical *ureA* genes [31].

In one patient, both *H. suis* and *H. pylori* was found. Similarly, other studies have shown presence of co-infections in human patients [26,30] and an increased prevalence of peptic ulceration during co-infection has been reported [26]. Indeed, in this study, the patient co-infected with *H. pylori* and *H. suis* showed presence of gastric ulceration, while the other patients infected with NHPH solely did not show presence of ulceration. Nevertheless, others did not detect differences in gastritis or ulceration in human patients infected with *H. pylori*, NHPH or both [30]. More patients should be investigated to verify if co-infections are associated with more severe gastric pathologies.

Detection of gastric NHPH infections is often hampered by the patchy and sparse colonization pattern of these bacteria in the human stomach. As a result, NHPH infections are easily missed by histopathological analysis and PCR, especially when only one or few gastric biopsies are examined [32]. In the present study, only one gastric biopsy was examined, which may have

183 resulted in a potential underestimation of the prevalence of gastric NHPH infections in patients
184 from Southern Turkey. In future studies, it would be interesting to investigate several gastric
185 biopsies taken from both the fundic and pyloric gland zone.

186 As *H. suis* naturally colonizes the stomach of pigs and non-human primates [12], while *H.*
187 *heilmannii* and *H. ailurogastricus* are mainly associated with dogs and cats [31], these animals
188 might function as a reservoir for human infections. MLST analysis demonstrated that the *H. suis*
189 strains from both patients were porcine associated [12]. The exact route of transmission from
190 animals to humans is not yet clear. Several studies showed that living in close proximity to as
191 well as intense contact with pigs, cats, and dogs leads to a significant risk of gastric NHPH
192 infection [33]. *Helicobacter* DNA has been detected in saliva from cats, dogs and pigs indicating
193 that the oral cavity of these animals may act as source of NHPH infection for humans [34–36].
194 Moreover, a pig veterinarian suffering from reflux oesophagitis and dyspepsia showed the
195 presence of *H. suis* colonization and inflammation. MLST analysis of *H. suis* revealed a very
196 close relationship with porcine *H. suis* strains, indicating that the infection originated through
197 close contact of the veterinarian with pigs [13,37]. Fecal-oral transmission has also been
198 suggested as a possible route for infection in cats [38]. Apart from direct contact, *H. suis*
199 presence was demonstrated on pork carcasses and in commercial pork, and the bacterium was
200 able to survive for at least 48h in minced pork [34,39], indicating that handling or consumption
201 of raw or undercooked pork might be a source of human infection as well. An additional
202 transmission route might be contaminated water, as *Helicobacter* spp. are able to survive in
203 water [40]. Finally, the role of wild mice as vector might be considered as well, since rodents are
204 easily colonized by most NHPH [4]. In this study, however, no information was obtained on
205 potential contact with infected animals and/or consumption of pork. Since it can be expected that

most patients were Muslims, contact with pigs or pork might be less likely. The source of infection with *H. suis* in the two patients from this study, thus remains unknown.

Gastroscopy of the NHPH infected children showed presence of hyperemia, nodular inflammation and/or ulceration. Furthermore, histopathological analysis revealed presence of an active chronic gastritis. This gives further evidence that infections with *H. suis* and *H. heilmannii/H. ailurogastricus* may be associated with the development of gastric disorders in children.

For the first time, presence of *H. pylori, H. suis* and *H. heilmannii/H. ailurogastricus* was shown in children suffering from gastric disorders in Southern Turkey. Apart from the well-known *H. pylori*, infection with NHPH may contribute to the development of gastric pathologies in children as well. Therefore, an increased awareness and diagnostic methods should be implemented to further clarify the epidemiology and pathology of these infections.

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Competing interest: The authors have no competing interests.

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Table 1: List of primers used in PCR to identify non-*Helicobacter pylori* helicobacter (NHPH) species present in the human stomach. All primers amplify part of the *ureA* gene.

Primer	Sequence	Amplicon size
Genus *Helicobacter*-forward	5′-CDGTRMGNTTTGARCCNGG -3′	250 bp
Genus *Helicobacter*-reverse	5′-GTDGTDGGDCCRTACATWGA -3′	250 bp
H. heilmannii-forward	5′-GGCTCTGCGTAGGMCCTGCTACRGAAGCTCTY -3′	110 bp
H. heilmannii-reverse	5′-GGCTGTRGGGATTTGTTGAGGAGARATG-3′	110 bp
H. suis-forward	5′-AAAACAMAGGCGATCGCCCTGTA -3′	150 bp
H. suis-reverse	5′-TTTCTTCGCCAGGTTCAAAGCG -3′	150 bp
H. bizzozeronii-forward	5′-AATCTTTGCGTGGGCCCTGCTACTGAGGCTTTG -3	130 bp
H. bizzozeronii-reverse	5′-CTGGCAAATGCTGTGGGGATTTGTTGG-3′	130 bp
H. felis-forward	5′-GCTGGTGGCATCGATACGCAT-3′	160 bp
H. felis-reverse	5′-TTTTTAGATTAGCGCGTCCGGGA-3′	160 bp
H. salomonis-forward	5′-CTCTTATGAGTTGGACTTGGTGCTCACCAAT-3′	125 bp
H. salomonis-reverse	5′-TTTGCCATCTTTAATTCCAATGTCGGC-3′	125 bp

D = A, G or T; R = A or G; M = A or C, N = A, G, C or T, W = A or T

Table 2: General overview of the presence of mucosal lesions (macroscopic evaluation during gastroscopy) and severity of gastritis (microscopic evaluation) in *Helicobacter* spp. PCR positive and negative children suffering from gastric disorders.

Macroscopic evaluation of gastric mucosal lesions	Absent	Hyperemia	Nodular	Ulcer
Total (n = 110)	90.0%	0.9%	6.4%	2.7%
Helicobacter spp. negative (n = 74)	98.6%	0%	1.4%	0%
Helicobacter spp. positive (n = 36)	72.2%	2.8%	16.7%	8.4%
H. pylori positive (n = 33)	75.8%	0%	16.6%	7.6%
H. heilmannii/H. ailurogastricus positive (n = 1)	0%	100%	0%	0%
H. suis positive (n = 1)	100%	0%	0%	0%
H. pylori + *H. suis* positive (n = 1)	0%	0%	100%	100%
Microscopic evaluation of the gastric inflammation	Absent	Mild	Moderate	Marked
Total (n = 110)	72.7%	8.2%	16.4%	2.7%
Helicobacter spp. negative (n = 74)	87.8%	1.4%	10.8%	0%
Helicobacter spp. positive (n = 36)	41.7%	22.2%	27.8%	8.3%
H. pylori positive (n = 33)	42.4%	24.2%	27.1%	6.1%
H. heilmannii/H. ailurogastricus positive (n = 1)	100%	0%	0%	0%
H. suis positive (n = 1)	0%	0%	0%	100%
H. pylori + *H. suis* positive (n = 1)	0%	0%	100%	0%

n = total number of investigated children. The data are shown as the percentage of children showing a certain lesion and degree of gastritis.