

***Helicobacter* species and gut bacterial DNA in Meckel's diverticulum and the appendix**

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Abstract

AIM: To analyse the possible association of various *Helicobacter* species and certain common gut bacteria in patients with Meckel's diverticulum and appendicitis.

METHODS: A nested-polymerase chain reaction (PCR), specific to 16S rRNA of the *Helicobacter* genus, was performed on paraffin embedded samples, 50 with acute appendicitis, 50 normal appendixes, and 33 Meckel's diverticulum with gastric heterotopia and/or ulcer. *Helicobacter* genus positive samples were sequenced for species identification. All samples were also analysed for certain gut bacteria by PCR.

RESULTS: *Helicobacter pullorum* DNA was found in one out of 33 cases and *Enterobacteria* in two cases of Meckel's diverticulum. *Helicobacter pylori* (*H. pylori*) was found in three, *Enterobacter* in 18, and *Bacteroides*

in 19 out of 100 appendix samples by PCR. *Enterococcus* was not found in any MD or appendix samples. All *H. pylori* positive cases were from normal appendixes.

CONCLUSION: *Helicobacter* is not an etiological agent in the pathogenesis of symptomatic Meckel's diverticulum or in acute appendicitis.

Key words: Meckel's diverticulum; *Helicobacter*; Appendix; Polymerase chain reaction

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INTRODUCTION

Although the stomach is the most frequent site of *Helicobacter pylori* (*H. pylori*) infection, *H. pylori* and enterohepatic *Helicobacter spp.* (EHS) have also been associated with extragastric diseases^[1].

Meckel's diverticulum (MD) is the most common developmental anomaly of the gastrointestinal tract and is present in 1%-2% of the general population. It often contains ectopic tissue, notably gastric and pancreatic tissue^[2]. Gastric mucosa is found in 10%-25% of MD and may be associated with inflammation, ulceration, gastrointestinal bleeding, and perforation^[3-5]. *H. pylori*

has been demonstrated in the ectopic gastric epithelium within MD^[6]. *Campylobacter-like* organisms in MD were first reported in 1989^[7-9]. However, conflicting results were reported concerning colonisation by *H. pylori* of such ectopic mucosa^[10,11]. There has been no study that investigated EHS in MD.

Acute appendicitis is the most common abdominal surgical emergency and can be seen in all ages, especially in those younger than 30 years^[12]. However, the aetiology of acute appendicitis is uncertain, and diagnosis is often difficult. There have been some investigations of *H. pylori* in appendix tissue^[13-15], but none that investigated non-*pylori Helicobacters*.

We hypothesized that non-*pylori Helicobacters*, such as enterohepatic *Helicobacters*, might be associated with these diseases. Most studies have investigated only *H. pylori* in MD and the appendix and mostly used non-molecular biological techniques; therefore, we aimed to analyse gastric, EHS and certain common gut bacteria in appendicitis and MD patients by genus specific polymerase chain reaction (PCR) and sequencing.

MATERIALS AND METHODS

Patients and histology

We re-examined all MD patients from 1990-2009 taken from the files of the Department of Pathology, Lund University Hospital. Thirty-three MD patients (two cases of ulcer without heterotopia, 31 cases with gastric heterotopia, of which seven also had an ulcer) (mean age: 11 years; range: 4 wk-73 years; 26 male, 7 female) were included in our study. Abdominal pain was the reason for operation in 16 cases, two of whom had acute appendicitis and one enlarged lymph nodes, nine were operated upon because of gastrointestinal bleeding and six for other abdominal diseases. No indication was given in two of the cases. Histological sections from stored paraffin blocks were stained with Alcian blue-periodic acid Schiff (AB-PAS) pH 2.5, Whartin-Starry silver stain and immunostained with an anti-*H. pylori* antibody (DAKO, Glostrup, Denmark, diluted 1:300).

We also re-examined mucosa from 50 cases of acute appendicitis (26 male, 24 female, median age: 30 years; range: 9-87 years) and 50 cases of normal appendix (16 male, 34 female, median age: 34 years; range: 10 d-80 years) from 2008-2009. Of the latter patients 26 were operated for a suspected appendicitis (8 male, 18 female, median age: 21 years; range: 8-77 years), 12 for intestinal diseases (8 male, 4 female, median age: 59 years; range: 10 d-80 years), and 12 for female genital disorders (median age: 38 years; range: 11-75 years). Histological sections from the *Helicobacter* positive cases were stained as mentioned above.

From the cases of MD, heterotopic mucosa of the gastric as well as the antral type were obtained from the paraffin blocks for PCR-assay with the tip of a scalpel. Ulcers were examined separately. In the case positive for *Helicobacter* DNA, the intestinal type mucosa surrounding

the heterotopia was also studied. Corresponding areas from appendix samples of mucosa, or of necrotic appendicitis were sampled for PCR. It was not possible to avoid including material from the appendical lumen in these samples. In the cases positive for *Helicobacter* DNA, other tissues removed at the same operation were also examined by PCR.

DNA extraction

DNA was extracted from the paraffin-embedded tissue samples by de-embedding, as previously described^[1]. DNA was extracted by a QIAamp DNA Mini Kit Tissue protocol (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Helicobacter specific PCR

DNA extracts were amplified in a GeneAmp 2700 Thermocycler (Applied Biosystems, Foster City, CA, United States) using a semi-nested PCR assay specific for *Helicobacter spp.* 16S rDNA, as previously described^[1]. *H. pylori* (CCUG 17874) was used as a positive control in all PCR reactions. The 416-bp PCR products were visualized by 1.3% agarose gel electrophoresis.

Amplification of non-Helicobacter bacteria

PCR specific for *Enterobacteriaceae*, the *Bacterioides-Prevotella* group, and *Enterococcus* were performed. The reaction mixture and amplification conditions, except for annealing temperatures, for non-*Helicobacter* PCR assays were the same as in the first step of the semi-nested *Helicobacter* PCR. The annealing temperatures and primers used for detection of *Enterobacteriaceae*, *Bacterioides-Prevotella* group, and *Enterococcus* were as described by Karagin *et al.*^[1] 2010. As positive controls, *Escherichia coli* (CCUG 17620), *Bacterioides fragilis* (CCUG 4856), and *Enterococcus faecalis* (CCUG 9997) were used in all PCR reactions. The 112-bp PCR product of *Enterococcus*, the 418-bp product of *Bacterioides*, and the 195-bp product of *Enterobacteriaceae* were visualized by 1.3% agarose gel electrophoresis.

DNA sequence analysis

Helicobacter specific PCR products were purified from agarose gels using the Montage DNA Gel Extraction Kit (Millipore, Bedford, MA, United States), according to the manufacturer's instructions. DNA sequence reactions were performed using the ABI PRISMTM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit version 3.0 (Applied Biosystems) as described by Tolia *et al.*^[16]. Products of the sequencing reaction were aligned and the closest homologous DNA was identified by BLASTn-analysis.

The study was approved by the Research Ethics Committee at Lund University, permit number 588/2006.

RESULTS

Histology

The most dominant heterotopia seen in MD was of the

corpus type with, in most cases, small areas of antral heterotopia. It was therefore easy to include both types of heterotopia, if present, in the same sample. No *Helicobacter* was found by histological and immunohistological examinations, neither in heterotopia nor in ulcers. In 3/33 of the MD cases, a mild chronic inflammation in the heterotopic area with slightly increased amounts of lymphocytes was seen. The ulcer was infiltrated with polymorphonuclear cells but there was no general, active gastritis. The normal intestinal mucosa in the MD outside the heterotopia did not have an increased amount of lymphatic tissue, in except the *Helicobacter pullorum* (*H. pullorum*) positive case. The nine ulcers and their surrounding mucosa were negative for *Helicobacter* DNA.

One of the heterotopic mucosa specimens was positive for *Helicobacter* DNA, namely that from a 44-year-old male. He was operated on for acute appendicitis. The appendix was not sent for histological analysis. The MD was also removed. Histology displayed a few gastric glands of the corpus type and a small strip of surface epithelium of the gastric type. There were a moderate number of lymphocytes and plasma cells in the heterotopic area. The surrounding mucosa of the intestinal type displayed an unusually well developed lymphatic tissue with germinal centres, a predominance of lymphocytes, and very few polymorphonuclear cells (Figure 1). There was no *Helicobacter* DNA detected by PCR in this sample.

Three normal appendixes were positive for *Helicobacter* DNA: from one an 18-year-old female with suspicion of appendicitis, one from a 63-year-old male with colon adenoma, one from a 55-year-old male with colon diverticulitis. Adenoma, diverticulitis, and normal colon tissue removed from the two latter patients were negative for *Helicobacter*. No tissue other than the appendix was removed from the first patient. All cases revealed *H. pylori* on sequence analysis. There was no gastric metaplasia in any of the appendixes, and no immunopositive *H. pylori* structures in the mucosa of the samples that were PCR-positive for *Helicobacter*.

Helicobacter specific PCR assay and sequencing results

Using the *Helicobacter* specific PCR assay and agarose gel electrophoresis, *Helicobacter spp.* was detected in 1/33 (3%) of specimens from patients with MD by genus specific nested-PCR. The sequenced PCR amplicon showed 98% similarity to *H. pullorum*. There were 3/50 (6%) samples that were positive for *Helicobacter spp.*, among normal appendixes. All of them showed 98%-99% sequence similarity to *H. pylori*. However *Helicobacter spp.* was not found in any samples of acute appendicitis.

PCR detection of bacterial DNA other than Helicobacter

Using the *Enterobacteria* specific PCR assay and agarose gel electrophoresis, *Enterobacteria spp.* was detected in 10/50 (20%) acute appendicitis cases and 8/50 (16%) normal appendixes. There were 7/50 (14%) and 12/50 (24%) samples that were positive for *Bacteroides spp.*,

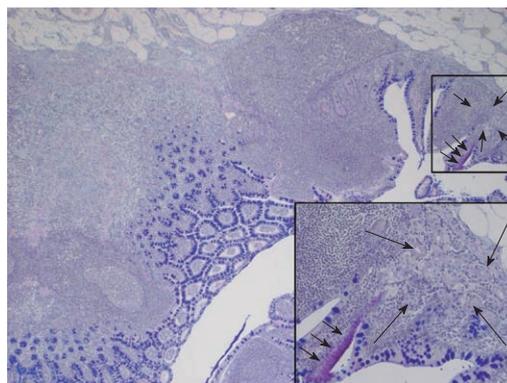


Figure 1 Microscopic analysis of the *Helicobacter pullorum* positive Meckel's diverticulum case. Low power view of a histological section from Meckel's diverticulum, positive for *Helicobacter pullorum* (*H. pullorum*) DNA, in the heterotopic area. There is an unusually well developed lymphatic tissue with germinal centres and a predominance of lymphocytes in the non-heterotopic area. Plasma cells were mainly found in the heterotopic area. The small arrows in the big square, point to a small strip of gastric heterotopia. This area displays at higher magnification in the inset. Large arrows point to gastric glands of corpus type. Blue without arrows in the photo indicates intestinal mucus. Alcian blue-PAS staining pH 2.5.

among the acute appendicitis and normal appendix sample, respectively. However, all MD and appendix samples were negative for *Enterococcus spp.* MD samples were also negative for *Bacteroides*; however, 2/33 (6%) were positive for *Enterobacteria spp.*

DISCUSSION

In this study, we screened for the presence of DNA of *Helicobacter spp.* and certain common intestinal bacteria by PCR in MD with gastric heterotopia and in appendix samples. We detected *H. pullorum* DNA in one out of 33 MD cases (3%) and *Enterobacteria* in two (6%). No *Enterococcus* or *Bacteroides* were found in the MD cases.

The *H. pullorum* case was positive only in the heterotopic area, not in the surrounding diverticulum mucosa of the intestinal type. No luminal contents were seen in these samples. This argues for the interpretation that the *H. pullorum* DNA originated from the heterotopic mucosa and not from the lumen. This assumption is further strengthened by the very low prevalence of other bacterial DNA in the MD samples. *H. pullorum* has, however, been described in stools from cases with gastroenteritis^[17], but our patient did not have such symptoms.

No *Helicobacter* was seen by immunohistochemistry. However, PCR is a more sensitive method and does not require intact bacteria. Our PCR technique is considered to be highly reliable for genus identification of *Helicobacter spp.*^[18,19]. Some authors have found *H. pylori* by immunohistochemistry in MD with active gastritis, implying the presence of polymorphonuclear cells; the prevalence varied between 2 and 28%^[6-8,20-23]. We had no cases with such inflammation and found no *H. pylori* DNA.

Interestingly, there was an increased amount of lymphatic tissue in the intestinal type mucosa of the *H.*

pullorum positive case. However, no conclusions can be drawn from just one case.

EHS are known to cause inflammatory bowel diseases^[24,25]. We have previously found *H. pullorum* DNA in cholecystitis samples with gastric metaplasia^[1]. Perhaps *H. pullorum* has some preference for the gastric epithelium.

We found *H. pylori* DNA in three out of 50 normal appendixes (6%) and none in the 50 cases of acute appendicitis. Other bacterial DNA was found in up to 24% of samples. We could not avoid including some luminal material in the appendix samples and therefore *H. pylori* DNA in the appendixes might be a contamination. Pavlidis *et al.*^[14] found *H. pylori* by PCR in two out of 46 samples (4%) of acute appendicitis. However, most authors have failed to demonstrate the presence of *H. pylori* in the appendix^[13,15,26]. *H. pylori* commonly colonises the gastrointestinal tract. However, our results suggest that *Helicobacter* is without importance in the etiology of acute appendicitis.

In conclusion, *H. pullorum* has, for the first time, been detected by PCR in MD patients with gastric heterotopias. However, there is no association between *H. pullorum* and MD pathogenesis. Moreover, *H. pylori* has no role in the aetiology of acute appendicitis. Its presence might have been that of a passenger.

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COMMENTS

Research frontiers

Helicobacter-like bacteria in Meckel's diverticulum (MD) have been reported by histological methods. However, no study has reported *Helicobacter* DNA in such specimens by polymerase chain reaction (PCR) and there is some doubt as to the presence of *Helicobacter* in patients with appendicitis.

Innovations and breakthroughs

Most studies have analyzed only *Helicobacter pylori* (*H. pylori*) in Meckel's diverticulum and appendix samples. However, enterohepatic *Helicobacter* species might also be important in the etiology of such diseases. The authors demonstrated the presence of *Helicobacter pullorum* in Meckel's diverticulum for the first time and concluded that *Helicobacter* might be a passenger in such patients.

Applications

By understanding the role of *Helicobacters* in the pathology of Meckel's diverticulum and appendicitis, this study could represent a future strategy for further pathological studies.

Terminology

Enterohepatic *Helicobacter* spp. (EHS) are the species of the genus *Helicobacter* that colonize the hepatobiliary tract and can cause extragastric diseases in humans or in animals.

Peer review

This work has been had the objective of seeking any association between *Helicobacter* species other than *H. pylori* with Meckel's diverticulum by very sensitive method, i.e., Nested PCR. Most previous studies were based on conventional methods, such as culture isolation, whereas in this study, the authors have used

molecular techniques. Although they did not find any association between MD and *Helicobacter* species, it does not undermine the importance of the study.

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