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Helicobacter pylori and inflammatory bowel diseases in tunisia

Abstract

Background/Aims: The prevalence of *Helicobacter pylori* (*H. pylori*) infection has been reported to be lower in individuals with inflammatory bowel disease (IBD) in both developing and developed countries. We investigated *H. pylori* infection in Tunisian patients with IBD and looked for possible associations of *H. pylori* infection and drug therapy for IBD. We also studied multiple and mixed infection by culture and antibiotic susceptibility methods and random amplified polymorphic DNA–PCR.

Methods: We studied 60 Tunisian IBD patients, including 45 CD (Crohn's disease) and 15 ulcerative colitis (UC) patients. Infection rates of *H. pylori* IBD patients as detected by serology were compared to control patients. *H. pylori* strains isolated from fundic and antral biopsies were studied by conventional culture methods. Two to nine single colonies per patient were subcultured so as to obtain pure isolates. Antimicrobial testing was performed for 25 strains. RAPD–PCR was conducted in 42 strains.

Results: The prevalence of *H. pylori* infection in the IBD patients was lower than the controls (53.3% vs 63.2%, p=0.960) whereas CD patients were observed to have the same rate as UC patients (p=0.618). There was a tendency for a higher frequency of lesions on the small bowel and colon in *H. pylori*–positive compared with *H. pylori*–negative CD patients (p=0.447*)*. A history of surgery seems to be more frequent in both *H. pylori*–negative CD patients (p=0.167) and *H. pylori*–positive UC patients (p=0.231*)*. For the medical treatment regimens shown, there was no significant difference between *H pylori* positive and negative. Mixed infection concerned metronidazole and ciprofloxacin in 1 patient while 1 patient showed a discordant susceptibility with regard to metronidazole. RAPD–PCR revealed that 4 of 5 patients displayed identical fingerprint profiles of strains, whereas one patient displayed 2 distinct fingerprint profiles.

Keywords: crohn's disease, *helicobacter pylori*, inflammatory bowel disease, rapd–pcr, Tunisia, ulcerative colitis

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Abbreviations: IBD: Inflammatory Bowel Disease; CD: Crohn's Disease; ELISA: Enzyme–Linked Immunosorbent Assay; CA– SFM: Antimicrobial Committee of the French society of Microbiology

Introduction

IBD comprises two main conditions: CD and UC. The aetiology of both conditions is poorly understood, but genetic, immunological and environmental factors all play a role.[1] CD is characterized by transmural inflammation of the gastrointestinal tract at any site from mouth to anus. UC affects only the mucosal layer of the gastrointestinal tract and extends in continuity from the rectum.[2] Like other developing countries, Tunisia has a high prevalence of *H. pylori* infection.[3,4] and a low prevalence of IBD, compared to developed countries.[5,6] *H. pylori* usually reside in the mucosa of the stomach but can also be found in the faecal stream and can be cultivated from the stool of infected individuals.[7] Recently there has been emerging epidemiological data to suggest that *H. pylori* may protect against certain IBDs such as CD and UC. However, the mechanism for the observed inverse association between *H. pylori* and IBD has not been described. The first observation that there was a negative association between *H. pylori* and IBD was made by El–Omar et al.[8] with the demonstration that *H. pylori* seropositivity was present in only 22% of IBD patients, but 52% of controls. The literature surrounding this curious association has recently been reviewed in detail by Luther et al.[9] including a meta–analysis of all published papers. The authors conclude that *H. pylori* seroprevalence is 27% in IBD patients *versus* 42% in control patients. It may be, therefore, that the relative immunosuppression initiated by *H. pylori* infection protects against other inflammatory gastrointestinal conditions such as IBD.[1]

The aim of our study was, first, to find out the prevalence of *H. pylori* infection in Tunisian IBD patients and to compare it to blood donors who had no history of gastric pathology; second, to identify any possible relation between *H. pylori* infection and a history of IBD treatment, and the phenotype of CD and UC; third, to study mixed and multiple infection in strains from gastric biopsies with culture and PCR–RAPD in *H. pylori* positive serology patient

Materials and methods

Study subjects

The study was conducted with IBD patients at the outpatient clinic of the Gastroenterology Unit A of Rabta Hospital in Tunis, Tunisia from April 2010 to May 2011. Of the 60 patients, 45 and 15 patients had CD and UC. They were 35 men and 25 women (gender ratio 1.4, mean age 39.1 years, range 16–67). The data concerning the various clinical parameters and treatment, including the timing of the onset of IBD symptoms and previous surgeries, were collected from patient documents and personal interview. The data of the control group was based on data of a previous report.[10] It consisted of Tunisian blood donors (212 male/38 female; gender ratio 0.18; mean age 33.5 years, range 25–55), seen as outpatients, who had no history of gastroduodenal disease and had come to the National Blood Transfusion Center in Tunis to make a blood donation.

H. pylori serology

Venous blood (5–10mL) was collected from all the enrolled subjects. Serum samples were obtained by centrifugation (3000 rpm for 10min), then aliquoted and immediately stored at –20°C until serology testing. The sera samples were investigated for IgG antibodies to *H. pylori* by an enzyme–linked immunosorbent assay (ELISA) technique, using a commercial kit (Platelia® *H. pylori* IgG de BioRad).

Biopsy specimens

Two antrum and two fundic biopsy specimens were collected from five IBD patients. Each biopsy was ground separately. Cultures were carried out on Columbia agar plates supplemented with 10% horse blood and Skirrow supplement (trimethoprim 5mg/L, vancomycin 10mg/L, polymyxin B 2500IU/L, Oxoid, France) at 37°C in microaerophilic conditions for 3–7 days (GENbox, BioMérieux, France). Depending on the number of colonies obtained in the primary culture, two to nine single colonies per patient (mean=4.5) were picked from the primary culture plates and were subcultured so as to obtain pure isolates. *H. pylori* isolates were identified as positive for urease activity and spiral shape morphology on Gram stain.

H. pylori genome typing by RAPD–PCR

DNA was extracted from colonies using the Qiagen DNA extraction kit (QIAamp DNA Mini Kit) according to manufacturer's instructions (Qiagen, Hilden, Germany). PCR–based RAPD fingerprinting was performed on 42 different strains as follows: the reaction was carried out in 50µl final volume containing 5µl of buffer, 4µl of MgCl2 (2mM), 5µl of dNTP (1mM), 2µl of primers, 0,5µl of Taq polymerase (Promega, France) (0,01U/µl) and 1µl of genomic DNA of *H. pylori*. A Perkin–Elmer 9700 thermal cycler (Applied Biosystems) was used for amplification. For each primer vil3 (5' GTTGGTGGCT3') and vil5 (5' AACGCGCAAC3'), the following cycling program was used:1 cycle of 94°C for 2 minutes, 38°C for 1 minute and 72°C for 4 minutes ; 29 cycles of 94°C for 2 minutes, 38°C for 3 minutes, 72°C for 7 minutes and then 72°C for 10 minutes. The PCR products were electrophoresed in 1.5% agarose gel after coloration with ethidium–bromide and photographed under UV light with a BioDoc Analyze system.

Multiple infection and mixed infection

Multiple infections were defined by different RAPD fingerprinting among the different isolates from a single patient. Mixed infection was defined by different resistance profiles among the different isolates from a single patient.[11]

Antimicrobial susceptibility testing

Strains were tested for metronidazole, clarithromycin and amoxicillin susceptibility using the E–test® (BioMérieux, France). Tetracycline, ciprofloxacin, erythromycin and rifampicin were tested using the disc diffusion method (Bio–Rad, France). Antimicrobial testing was performed on Columbia agar with 5% horse blood agar according to the CA–SFM (Antimicrobial Committee of the French society of Microbiology) 2010 recommendations. Inoculum was prepared from 2–day–old agar plates, colonies were harvested in 2mL of Brucella broth. The final inoculum was adjusted to 3 McFarland turbidity standard (approximately 10^8CFU/mL) and was spread on agar plates. Minimal inhibitory concentration cut–offs to define resistance were 0.12mg/L for amoxicillin, 0.25/0.50mg/L for clarithromycin and 8mg/L for metronidazole.

Statistics

Categorical data were analysed by the Chi2 test and Fisher's exact test. p–values lower than 0.05 were considered statistically significant.

Results

Patient characteristics

Prevalence of *H. pylori* infection in IBD patients and in controls is detailed in Table.1. The prevalence of *H. pylori* infection in the IBD patients was lower than the controls (53.3% vs 63.2%, p=0.960) whereas CD patients were observed to have the same rate as UC patients (p=0,618), but it wasn't statistically significant. Also, we did not find any meaningful associations of *H. pylori* infection rate with gender or age (Table 1).

Table 1 Clinical Characteristics of the IBD Patients and Blood donors

	IBD (n=60)	CD (n=45)	UC (n=15)	Blood Donors (n=250)	P Value
Male /Female	35/25	29/16	9-Jun	212/38	0.087
Mean Age (Years)	39.7	39.2	40.2	33.5	0.774
Prevalence of Hp+ (%)	32 (53.3%)	24 (53.3%)	8 (53.3%)	158 (63.2%)	0.96

IBD, Inflammatory Bowel Disease; CD, Crohn's Disease; UC, Ulcerative Colitis; *Hp+*, *H. pylori* Seropositive

H. pylori infection and location of lesions of IBD

The location of gastroduodenal lesions at diagnosis of disease and surgery were grouped by *H. pylori* serology status for CD and UC respectively (Table 2 & 3). There was a tendency for a lower frequency of lesions on the small bowel in *H. pylori*–positive compared with *H. pylori*–negative CD patients, which did not reach statistical significance (p=0.047), but a higher frequency of lesions on the colon. *H. pylori*–positive UC patients tended to have a lower incidence of pancolitis involvement (Table 3). The number of patients wasn't statically significant (p=0.070). Surgery seems to be more frequent both in *H. pylori*–negative CD patients (63.2%, p=0.167) and in *H. pylori*–positive UC patients (28.6%, p=0.231). It wasn't statistically significant.

Table 2 *H. pylori* infection and site of disease at diagnosis of CD

Site of Disease at Diagnosis	*H. pylori* Positive (n=24)	%	*H. pylori* Negative (n=21)	%	P Value
Small Bowel	24-Jul	29.2	21-Sep	42.9	
Colon	24-Jul	29.2	21-Mar	14.3	0.447
Small Bowel and Colon	24-Sep	37.5	21-Sep	42.9	
Perianal Disease Involvement	24-Jan	4.2	0/21	0	

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Table 3 *H. pylori* infection and phenotype of UC

	H. pylori Positive (n=8)	%	*H. pylori* Negative (n=7)	%	P Value
Left Colitis	8-Mar	37.5	0/7	0	
Pan Colitis	8-Feb	25	7-Jun	85.7	
Rectosigmoiditis	8-Mar	37.5	0/7	0	0.07
Rectitis	0/8	0	7-Jan	14.3	

H. pylori infection and a history of IBD treatment

Medical treatment for CD and UC are presented respectively in Table 4 & 5. For the medical treatment regimens shown, there was no significant difference between *H. pylori* positive and negative groups.

Table 4 Prevalence *H. pylori* infection in the CD patients according to drug history

CD Treatment	HP+ (n=24)	%	HP- (n=21)	%	P Value
Salazopyrine	8	33.3	5	23.8	0.356
5-ASA[a]	5	20.8	5	23.8	0.546
Corticosteroids	14	58.3	14	66.7	0.396
Immunosuppressive Therapy	18	75	17	81	0.454
Infliximab	5	20.8	5	23.8	0.546
Antibiotics[b]	14	58.3	9	42.9	0.231

a :5-Aminosalicylic Acid; **b**: Antibiotics administrated were ciprofloxacin, metronidazole, gentamicin, isoniazid, cefotaxime or rifampicine

Table 5 Prevalence *H. pylori* infection in the UC patients according to drug history

UC treatment	HP+ (n=8)	%	HP- (n=7)	%	P value
Salazopyrine	6	75	2	28.6	0. 100
Mésalazine	3	37.5	4	57.1	0..405
Corticosteroids	6	75	6	85.7	0.554
Immunosuppressive Therapy[a]	3	37.5	3	42.9	0.622
Antibiotics[b]	4	50	3	42.9	0.595

a: Immunosuppressive therapy consisted of methotrexate, azathioprine or cyclosporine; **b**: Antibiotics administrated were ciprofloxacin, metronidazole, gentamicin, isoniazid, cefotaxime or rifampicine

Gastric endoscopy

Eight positive *H. pylori* serology patients with IBD underwent an upper gastrointestinal endoscopy (5 male, 3 female, mean age: 40). Biopsy samples were taken from the gastric antrum and the pylorus in separate tubes for 7 patients. For one patient, antral and fundic biopsies were in the same tube. We received fifteen biopsies: 10/15 (66.6%) were urease positive, 8/15 (53.3%) of the gram staining was presumptive of *H. pylori* and 8/15 (53.3%) had a positive culture.

Antibiotic susceptibility testing

Between 2 and 9 separate colonies were isolated from each biopsy of 5 *H. pylori*–positive CD patients. Each colony was subcultured in a single plate and antibiotic susceptibility was performed on each colony (n=25). Antibiotic susceptibility was performed on 25 strains. Only 21.7% (5/23) of the isolates were resistant to metronidazole (MIC values 32–256mg/L). Resistance to metronidazole was detected in 4 patients in whom 2 patients had received this antibiotic for the IBD. Two strains from one patient who had never received metronidazole had different MIC values (32 and 128mg/L). It was a mixed infection. Sixteen per cent of the strains (4/25) were resistant to ciprofloxacin by disc method in 4 antral strains (4/25). Resistance concerned a single patient who had never received this antibiotic for IBD treatment. Furthermore, all the isolates were found to be highly sensitive to amoxicillin, clarithromycin by the E–test method. The isolats were sensitive to Erythromycin, tetracycline and rifampicin by disc diffusion method. Mixed infection concerning two different antibiotics (metronidazole and ciprofloxacin) was detected in 1 patient while 1 patient showed a discordant susceptibility against metronidazole.

RAPD–PCR analysis

RAPD–PCR fingerprinting was carried out on the paired isolates from 5 patients. RAPD–PCR was performed for 42 strains (range 2–9 strains per patient). RAPD–PCR revealed that 4 of 5 patients displayed identi were sensitive cal fingerprint profiles of strains, whereas one patient displayed 2 distinct fingerprint profiles. Two different patterns were observed among the isolates of patient N°5 indicating a multiple infection whereas antibiotic susceptibility was identical for these strains. Two different patterns were observed among the isolates of one patient, N°5 (Figure 1).

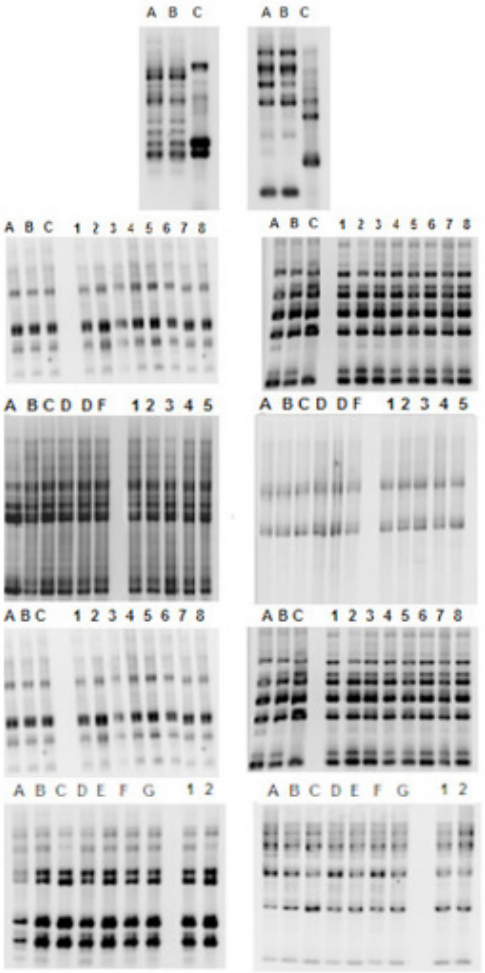

Figure 1 RAPD-PCR fingerprints of *H. pylori* isolates (antral and fundic) from 5 patients (N°1-5) using RAPD primers vil 3 (images on the right) and vil 5 (images on the left). The above fingerprints demonstrate that the isolates in Patients 1. 2. 3 and 4 are the same strains as they display identical antral and fundic patterns. Lanes A to I represent antral strains. Lanes 1 to 8 represent fundic strains. Two different patterns were observed among the isolates of one patient N°5 (Lane C).

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Discussion

To our knowledge, our study is the first report of *H. pylori* seroprevalence in IBD in Tunisia. Our results confirm the lower prevalence of *H. pylori* infection in IBD patients found elsewhere. They are in concordance with numerous reports reported in both developing and developed countries.[8,11–16] It appears in Puspok's study that in *H. pylori*–infected patients the disease is more often confined to the ileum.[17] whereas we noticed a higher frequency of lesions on the small bowel and colon in *H. pylori*–positive CD patients compared with those who were *H. pylori* negative, a result which did not reach statistical significance. A hypothesis for how *H. pylori* may influence the clinical course of IBD is that the host response to *H. pylori* infection is localized mainly in the stomach. However, activated lymphocytes of the MALT are able to lodge anywhere in the GI tract and therefore may lead to a more generalized immune response to *H. pylori* infection throughout the gastrointestinal tract in inflammatory bowel disease. Alternatively, the MALT of the intestine itself may be directly activated by *H. pylori* or its antigens passing with the stool.[18] In our study, a history of surgery seems to be more frequent in *H. pylori*–negative CD patients (p=0.167) whereas Puspok found that it was more frequent in *H. pylori*–positive CD patients.[17]

Another aim of the study was to assess the possible effect of medical treatment of IBD on *H. pylori* seropositivity. Treatment with 5–aminosalicylic acid, azathioprine and corticosteroids was not related to *H. pylori* seropositivity. Corticosteroids suppress epithelial proliferation, which is thought to render the mucosa susceptible to the effects of ulcerogens. However, in our study there was no significant difference in *H pylori* status between patients who were taking steroids and those who were not.[14] We presumed that this lower infection rate in CD patients might be due to the frequent and prolonged use of antibiotics to treat abscesses or anal fistulas.

The association was attributed to sulphasalazine use, a finding that has been supported by other authors.[12,13] Subsequent work has demonstrated that the difference in prevalence appears independent of sulphasalazine use.[19,20] Among several genotyping methods applied to *H. pylori*. RAPD–PCR is considered to be useful because it is a simple, rapid and low–cost means of distinguishing *H. pylori* isolates.[21] One interesting aspect of the study is the fact that mixed infection (susceptible and resistant isolates) occurs mainly among patients with a single infection (unique RAPD fingerprint) (80%). Indeed, 4 patients with mixed infection showed identical fingerprinting patterns thereby suggesting an infection with a single *H. pylori* strain. In one patient's strains, we observed detectable differences in the DNA pattern were clear thereby suggesting multiple infections. In Ben Mansour et al.[11] study, the prevalence of multiple infection estimated in Tunisian patients was 48% compared to 5% respectively among French patients. As is the case in many other developing countries, the prevalence of *H. pylori* infection in Tunisia is high and the chances for a single Tunisian host to be infected or re–infected by different strains are greater than they are in France, a developed country where prevalence is low.

Antibiotic susceptibility was performed on 25 strains. Only 21.7% (5/23) of the isolates were resistant to metronidazole. 16% of the strains (4/25) were resistant to ciprofloxacin. The results didn't reach significance because of the limited number of biopsies. In fact, our study coincided with major political events in Tunisia in 2011. The majority of patients had difficulty reaching our clinic for consultation, despite reminder phone calls. This finding is in agreement with previous studies showing that DNA fingerprinting patterns of different isolates with different susceptibilities from a single patient are identical, which may mean that antibiotic–resistant *H. pylori* strains typically develop from a pre–existing susceptible strains rather than from co–infection with different strains.[22,23]

Conclusion

The causes of IBD are still unclear. Several hypotheses have been put forward, some of which have attributed a role to *H. pylori* infection in the pathogenesis of IBD. Several serological and molecular studies have been carried out in this direction. More detailed studies will be needed in the future to shed light on issues that remain unresolved, such as the question of whether IBD is influencing *H. pylori* infection or vice versa.

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Conflicts of interest

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