Does *Helicobacter Pylori* Identification in the Mucosa of the Gallbladder Correlate with Cholesterol Gallstone Formation?

J Griniatsos[1], S Sougioultzis[2], K Giaslakiotis[3], M Gazouli[4], E Prassas[1], E Felekouras[1], O Michail[1], E Avgerinos[1], E Pikoulis[1], G Kouraklis[1], I Delladetsima[3], M Tzivras[2]

ABSTRACT

Objective: Helicobacter pylori (H pylori) *represents a potential initiator of cholesterol crystallization and it has been proposed that it is related to gallstone formation. In this study, any possible association between the* H pylori *identification in the mucosa of gallbladder and cholesterol gallstone formation was evaluated.*

Methods: *Gallbladders containing pure or mixed cholesterol gallstones (cholelithiasis group, n = 89) and gallbladders without gallstones (control group, n = 42) were submitted to standard histopathological examination for* H pylori *detection, as well as to nested polymerase chain reaction amplification for* H pylori *DNA detection.*

Results: Helicobacter pylori *was identified in the gallbladder's epithelium in four patients with cholelithiasis and in two patients in the control group by histology. In all the cases which were found to be* H pylori *positive by histological examination,* H pylori *DNA were also detected. No correlation between gallstone formation and* H pylori *detection in the biliary epithelium was found. A higher incidence of acute inflammation in the cholelithiasis (22.5% vs 9.5%,* p = *not significant [ns]) and in the* H pylori *positive groups (33% vs 17.6%,* p = *ns) were histologically detected. A higher incidence (10% vs 0%),* p = *ns) of* H pylori *in gallbladders with gallstones and acute inflammation, compared to gallbladders with acute inflammation but without gallstones, was noticed.*

Conclusion: Helicobacter pylori *is detectable in low frequency in the mucosa of the gallbladder and it does not seem to act as a lithogenic component for cholesterol gallstone formation. Its higher incidence in gallbladders with gallstones and acute inflammation, suggests a possible accessory role in a subset of patients with cholelithiasis.*

¿Existe una Correlación de la Identificación de *Helicobacter Pylori* en la Mucosa de la Vesícula Biliar con la Formación de Cálculos Biliares de Colesterol?

J Griniatsos[1], S Sougioultzis[2], K Giaslakiotis[3], M Gazouli[4], E Prassas[1], E Felekouras[1], O Michail[1], E Avgerinos[1], E Pikoulis[1], G Kouraklis[1], I Delladetsima[3], M Tzivras[2]

RESUMEN

Objetivo: Helicobacter pylori (H pylori) *representa un iniciador potencial de la cristalización del colesterol, y se ha propuesto que guarda relación con la formación del cálculo biliar. En este estudio, se evaluó cualquier posible asociación entre la identificación de* H pylori *en la mucosa de la vesícula y la formación del cálculo biliar de colesterol.*

Métodos: *Las vesículas que contienen cálculos biliares de colesterol puros o mixtos (grupo de colelitiasis, n = 89) y vesículas sin cálculos biliares (grupo control, n = 42) fueron sometidos a un examen histopatológico estándar con el fin de detectar el* H pylori *descubrimiento, así como a la amplificación de la reacción en cadena de polimerasa para la detección de ADN H pilori.*

Resultados: *El Helicobacter pylori fue identificado mediante histología en el epitelio de la vesícula en cuatro pacientes con el colelitiasis y en dos pacientes en el grupo de control. En todos los casos que*

From: [1]First Department of Surgery, [2]Gastroenterology Unit, Department of Pathophysiology, [3]Pathology Department, [4]Department of Biology, University of Athens, Medical School, "Laiko" Hospital, GR 115-27, Athens, Greece.

Correspondence: Dr J Griniatsos, Assistant Professor of Surgery, 17 Agiou Thoma str, G R 115-27, Athens – GREECE, Fax: 0030 210 7771195, e-mail: johngriniatsos@yahoo.com.

resultaron ser H pylori *positivo por el examen histológico, se halló también DNA* H pylori. *No se halló correlación ninguna entre la formación del cálculo biliar y la detección de* H pylori *en el epitelio biliar. Se detectó histológicamente una incidencia más alta de inflamación aguda en la colelitiasis (22.5% contra 9.5%,* p = *no significativo [ns]) y en los grupos* H pylori *positivos (33% contra 17.6%,* p = *ns). Se observó una incidencia más alta (10% contra 0%),* p = *ns) de* H pylori *en las vesículas con los cálculos biliares e inflamación aguda, en comparación con las vesículas con la inflamación aguda pero sin cálculos biliares.*

Conclusión: Helicobacter pylori *es detectable en baja frecuencia en la mucosa de la vesícula y no parece actuar como un componente litogénico en la formación del cálculo biliar de colesterol. Su mayor incidencia en las vesículas con cálculo biliar e inflamación aguda, hace pensar en un posible papel auxiliar en un subconjunto de pacientes con colelitiasis.*

West Indian Med J 2009; 58 (5): 429

INTRODUCTION

Helicobacter pylori (*H pylori*) is implicated in the pathogenesis of gastric and duodenal ulcer (1) and has been proposed as a risk factor for gastric cancer development (2, 3). *Helicobacter pylori* infection has also been associated with various extragastric diseases, including lesions of the gallbladder (4). Kawaguchi *et al* (5) first reported the presence of bacteria resembling *H pylori* in the gallbladder's mucosa of a patient with gallstone cholecystitis and suggested a contribution between infection and gallstone formation. Following this initial report, *H pylori* has been identified in the bile (6–8), the liver (9) and the biliary epithelium (10, 11) of humans and its presence has been proposed as related to several hepatobiliary diseases. There is little doubt that bacteria, including *H pylori*, play a significant role in pigment gallstone formation (12). Bacterial genetic material has also been identified in pure and mixed cholesterol gallstones (13). Although, recent animal studies did not confirm any association between *H pylori* infection and cholesterol gallstone formation (14), clinical studies have reached contradictory results, providing data in favour of (7, 10, 15) or against (6, 11, 16–18) the possible role of *H pylori* as predisposing factor for cholesterol gallstone formation.

There is a high prevalence of gallstone disease in Greece (19) and a high *H pylori* seroprevalence, ranging from 40% in age groups younger than 40 to 77% in those older than 60 years (20).

The aim of the present prospective case – control study was to investigate any possible association between *H pylori* identification in the gallbladder mucosa and cholesterol gallstone formation in a subset of patients with cholelithiasis.

MATERIAL AND METHODS

The examined material consisted of 89 gallbladders containing one or more pure or mixed cholesterol gallstones. They originated from 22 male and 67 female patients with median age of 63 years (Interquartile Range, IR 51–73) who underwent cholecystectomy between April 2006 and March 2008 due to symptomatic gallstone disease. Among them, ten patients (n = 10) had emergency operations due to acute cholecystitis, unresolved after 48 hours conservative management with antibiotic administration. Patients with pigment gallstones or gallbladder polyps, as well as patients with a history of previous gastric surgery were excluded from the study.

Forty-two (n = 42) gallbladders without cholelithiasis constituted the control group. These were from patients with a median age of 58 years (IR 52–67) who underwent Whipple's operation (n = 13) or pylorus-preserving pancreatoduodenectomy (n = 7) for pancreatic head cancer, as well as from patients who underwent different types of liver resection for hepatocellular carcinoma (n = 8) or metastatic colorectal cancer to the liver (n = 14).

The prevalence of *H pylori* infection in the population of the study was not different from its prevalence in the general Greek population, based on the origin, the gender and the age of the enrolled patients, as well as the current epidemiological data from Greece.

The study was conducted according to the declaration of Helsinki and informed consent was obtained from all patients. Gallstone subtyping was performed routinely by gross inspection after submission to the Pathology Department (21).

Gallbladder specimens were fixed in 10% buffered formalin solution and sampling was performed following established guidelines. Paraffin sections were stained with haematoxylin and eosin according to routine protocol. Screening for *H pylori* was carried out on Giemsa stain by two pathologists under an optical microscope (Olympus® BH2) while their presence was confirmed by additional silver stain (Warthin-Starry). Histological findings were grouped in two main diagnostic categories defined as chronic and acute on chronic cholecystitis. Acute inflammation was characterized by the presence of neutrophils.

H pylori DNA – PCR amplification. After the accomplishment of histopathological examination, the paraffin blocks were further submitted to polymerase chain reaction (PCR) amplification for *H pylori* DNA detection. In all cases, including those of the control group, DNA was extracted from the formalin-fixed paraffin-embedded (FFPE) gallbladder tissue using the NucleoSpin Tissue Kit

(Macherey-Nagel, Germany), according to manufacturer's instruction. To confirm the integrity of DNA, a 430 bp sequence in the human glyceraldehyde-3-phosphatate dehydrogenase (GAPDH) gene was amplified.

Nested PCR for *H pylori* was performed using primers of the 26-kDa species-specific antigen SSA gene (22, 23). Specifically, we used the outer-1 primer: 5'-TGGCGTGTCTATTGACAGCGAGC- 3'and outer – 2 primer: 5'-CCTGCTGGGCATACTTCACCAT -3' that produced a PCR fragment of 303 bp, and the internal primers, internal-1: 5'-GAAAAAGGCGGTATCGGTCA-3' and internal-2: 5'-CAATCAAAAAAGCACCTCTCAAAG-3' produced the final PCR fragment of 121 bp. The first PCR was performed under the following conditions: at 95°C for 5 min, followed by 35 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec and a final extension at 72°C for 5 min. The second PCR was performed using as a template 1 μL of the first PCR reaction product. Cycling conditions of the second PCR were the same with the first ones, except the annealing temperature at 52°C for 30 sec. The amplification products were analyzed by electrophoresis in ethidium bromide-stained 2% agarose gel. Negative and positive controls were assayed in each experiment.

Univariate analyses between groups were determined by the Fisher exact test. $p < 0.05$, was considered statistically significant. All statistical calculations were performed using the SPSS (version 12-0) statistical programme.

RESULTS

In the examined material, the overall incidence of *H pylori* presence in the gallbladder mucosa was 4.5%. In particular, *H pylori* was identified by Giemsa stain (Fig. 1A) in four cases of cholelithiasis (4/89) and in two cases of the control group (2/42). In all six cases, *H pylori* presence was confirmed by the Warthin-Starry silver stain (Fig. 1B). *Helicobacter pylori* micro-organisms were encountered locally and were usually low in number.

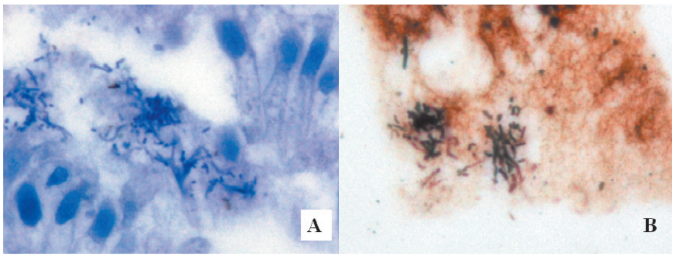

Fig. 1A: *Helicobacter pylori* as shown in Giemsa stain.
1B: *Helicobacter pylori* as shown in Warthin-Starry stain.

Helicobacter pylori DNA was identified by PCR amplification in 6 out of the 131 cases; four in the cholelithiasis and two in the control group (Fig. 2). There was an exact match between the molecular and histologic findings; in all cases which were found to be *H pylori* positive by histological examination, *H pylori* DNA was also detected.

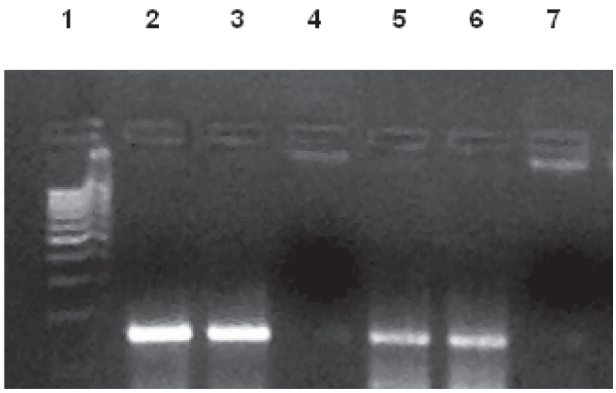

Fig. 2: Representative results of PCR.
Lane 1: 100bp DNA Ladder (Fermentas)
Lanes 2, 3, 5: Samples positive for *H pylori*
Lane 4: Sample negative for *H pylori*
Lane 6: Positive control
Lane 7: Negative control sample without DNA

As shown in Table 1, no correlation between gallstone formation and *H pylori* detection in the biliary epithelium was found. In the cholelithiasis group, the presence of *H pylori* correlated neither to the total number nor to the size of the gallstones (not shown). Although not statistically significant ($p = 0.074$), a higher incidence of acute inflammation in the cholelithiasis group (22.5%), compared to the control one (9.5%), was histologically detected. Moreover, *H pylori* was detected in 2/20 (10%) patients with gallstones and acute inflammation. On the contrary, *H pylori* was not detected in any of the gallstone-free gallbladders with acute inflammation.

Table 1: Epidemiological characteristics of the two groups of patients

Parameter		Cholelithiasis group (n = 89)	Control group (n = 42)	*p value*
Histology positive for HP		4	2	ns
PCR positive for HP		4	2	ns
Sex	Female	67	26	
	Male	22	16	ns
Age (years)	Median + IR	63 (51 – 73)	58 (52 – 67)	ns
Acute inflammation in histology	Present	20	4	
	Absent	69	38	0.074

Comparative analysis between the *H pylori* (+) and *H pylori* (-) patients (Table 2) disclosed a marginally statistically significantly ($p = 0.049$) higher incidence of *H pylori* in patients over 70-years old. A higher incidence of neutrophil infiltration in the *H pylori* (+) group (33%), compared to the *H pylori* (-) group (17.6%) was histologically detected, but the difference did not reach statistically significant levels ($p = 0.334$). *Helicobacter pylori* was not identified in the gallbladder mucosa, by either method, in any of the ten patients with clinically acute cholecystitis.

Table 2: Univariate analysis between HP (+) and HP (–) patients

	Parameter	HP (+) (n = 6)	HP (-) (n = 125)	*p value*
Sex	Male	1	37	
	Female	5	88	ns
Age (years)	Median + IR	73 (60 – 75)	60 (50 – 72)	ns
Age	> 70 years	4	36	
	≤ 70 years	2	89	0.049
Etiology	Cholelithiasis	4	85	
	Other disease	2	40	ns
Indication for operation				
	Symptomatic cholelithiasis	4	75	ns
	Other disease	2	40	ns
	Acute cholecystitis (n = 10)	0	10	ns
Acute inflammation in histology				
	Present	2	22	
	Absent	4	103	ns

DISCUSSION

In the present study, the overall incidence (4.5%) of the *H pylori* presence in the gallbladders' specimens was low and no differences were found between patients with cholesterol gallstones and patients without cholelithiasis.

The presence and identification of the *H pylori* was confirmed by using a specific nested PCR that targets the 26-kDa SSA gene, a method that has been reported as the most appropriate for *H pylori* detection in gastric biopsy specimens (23). Polymerase chain Reaction (PCR) testing also contributed in the reliability control of the histochemical special stains for *H pylori* which were proven to be of high sensitivity and specificity as in the gastric mucosa.

Data in this study , showing a low incidence of *H pylori* colonization of the biliary mucosa, is in agreement with similar studies from different countries such as Canada (6), Turkey (15), Mexico (16) and Germany (17, 18). On the other hand, it is in contrast with findings from other populations such as Serbians (10), Italians (7, 24), Brazilians (25), Chileans (26) and Ukrainians (27). The latter studies, following different investigational approaches, concluded that the incidence of *H pylori* in the biliary tree is as high as 60%. This broad variation in the colonization rate of the biliary system cannot be explained only by the differences in the *H pylori* seroprevalence among different populations. Methodological and experimental variability, as well as differences in sensitivity and specificity of the applied molecular techniques used for the detection of bile-resistant *Helicobacter* species (18) have to be taken into consideration.

Several investigators (24, 25, 28) proposed that colonization of the mucosa of the gallbladder by *H pylori* is a potential risk factor for gallstone formation. The background theory is that colonization of the mucosa by *H pylori*, as is the case with other bacteria, may cause chronic inflammation, impairing the acid secretion, reducing the solubility of calcium salts in the bile and increasing the risk of their precipitation in the lumen, thus favouring gallstone formation (29). The bacteria in pure and mixed cholesterol gallstones were located in the centre of the stone, suggesting that they facilitate formation of nidus and possible cholesterol precipitation (13).

Data from the present study strengthen the notion that *H pylori* species are not a major contributor to pure and mixed cholesterol gallstone formation, since there was no difference in the colonization rate of the gallbladder mucosa between patients with and without cholelithiasis. However, the higher incidence of *H pylori* in gallbladders with gallstones and acute inflammation, compared to gallbladders with acute inflammation but without gallstones (10% *versus* 0%), suggests a possible accessory role in a subset of patients with cholelithiasis.

The finding of the present study shows that no *H pylori* was identified among the 10 patients with clinically acute cholecystitis, could be related not only to the therapeutic effect of the antibiotic therapy given to all these patients before cholecystectomy, but also to the overall low incidence of *H pylori* presence in the gallbladder mucosa of this studied population as well as the central role of other bacteria in the acute inflammation process.

Moreover, a relative increased frequency of *H pylori* colonization was found in the gallbladders of patients older than 70 years, a finding which may be explained by the increased prevalence of *H pylori* infection in this age group (30), raising the possibility for *H pylori* colonization of the biliary tree from the stomach.

According to our findings, *H pylori* is detectable in a low frequency in the gallbladder mucosa and it does not seem to act as a lithogenic component in the context of pure or mixed cholesterol gallstone formation. The relative higher frequency of *H pylori* in the biliary mucosa of patients older than 70 years, probably reflects the increased prevalence of the infection in this age group.

REFERENCES

1. Kuipers EJ, Thijs JC, Festen HP. The prevalence of Helicobacter pylori in peptic ulcer disease. Aliment Pharmacol Ther 1995; 9 (**Suppl 2**): 59–69.
2. Sougioultzis S, Foukas PG, Tzivras M, Kourtessas D, Gorgoulis VG, Davaris P et al. Alterations in the proliferating compartment of gastric mucosa during *Helicobacter pylori* infection: the putative role of epithelial cells expressing p27 (kip1). Mod Pathol 2003; **16:** 1076–85.
3. Huang JQ, Sridhar S, Chen Y, Hunt RH. Meta-analysis of the relationship between Helicobacter pylori seropositivity and gastric cancer. Gastroenterology 1998; **114**; 1169–79.
4. Goodman KJ, Joyce SL, Ismond KP. Extragastric diseases associated with Helicobacter pylori infection. Curr Gastroenterol Rep 2006; **8:** 458–64.
5. Kawaguchi M, Saito T, Ohno H, Midorikawa S, Sanji T, Handa Y et al. Bacteria closely resembling Helicobacter pylori detected immunohistologically and genetically in resected gallbladder mucosa. J Gastroenterol 1996; **31:** 294–8.
6. Fallone CA, Tran S, Semret M, Discepola F, Behr M, Barkun AN: Helicobacter DNA in bile: correlation with hepato-biliary diseases. Aliment Pharmacol Ther 2003; **17:** 453–8.

7. Figura N, Cetta F, Angelico M, Montalto G, Cetta D, Pacenti L et al. Most Helicobacter pylori-infected patients have specific antibodies, and some also have H. pylori antigens and genomic material in bile: is it a risk factor for gallstone formation? Dig Dis Sci 1998; **43:** 854–62.
8. Lin TT, Yeh CT, Wu CS, Liaw YF. Detection and partial sequence analysis of Helicobacter pylori DNA in the bile samples. Dig Dis Sci 1995; **40:** 2214–9.
9. Nilsson HO, Taneera J, Castedal M, Glatz E, Olsson R, Wadstrom T. Identification of Helicobacter pylori and other Helicobacter species by PCR, hybridization, and partial DNA sequencing in human liver samples from patients with primary sclerosing cholangitis or primary biliary cirrhosis. J Clin Microbiol 2003; **38:** 1072–6.
10. Bulajic M, Maisonneuve P, Schneider-Brachert W, Muller P, Reischl U, Stimec B et al. Helicobacter pylori and the risk of benign and malignant biliary tract disease. Cancer 2002; **95:** 1946–53.
11. Myung SJ, Kim MH, Shim KN, Kim YS, Kim EO, Kim HJ *et al*. Detection of Helicobacter pylori DNA in human biliary tree and its association with hepatolithiasis. Dig Dis Sci 2000; **45:** 1405–12.
12. Stewart L, Oesterle AL, Erdan I, Griffiss JM, Way LW. Pathogenesis of pigment gallstones in Western societies: the central role of bacteria. J Gastrointest Surg 2002; **6:** 891–903.
13. Stewart L, Grifiss JM, Jarvis GA, Way LW. Biliary bacterial factors determine the path of gallstone formation. Am J Surg 2006; **192:** 598–603.
14. Maurer KJ, Rogers AB, Ge Z, Wiese AJ, Carey MC, Fox JG. Helicobacter pylori and cholesterol gallstone formation in C57L/J mice: a prospective study. Am J Physiol Gastrointest Liver Physiol 2006; **290:** G175–82.
15. Abayli B, Colakoglu S, Serin M, Erdogan S, Isiksal YF, Tuncer I et al. Helicobacter pylori in the etiology of cholesterol gallstones. J Clin Gastroenterol 2005; **39:** 134–7.
16. Méndez-Sánchez N, Pichardo R, González J, Sanchez H, Moreno M, Barquera F et al. Lack of association between Helicobacter sp colonization and gallstone disease. J Clin Gastroenterol 2001; **32:** 138–41.
17. Roosendaal R, Kuipers EJ, Vandenbroucke-Grauls CM, Kusters JG. Helicobacter species are not detectable by 16S rDNA PCR in bile from Dutch patients with common bile duct stones. Digestion 2002; **66:** 89–91.
18. Rudi J, Rudy A, Maiwald M, Stremmel W. Helicobacter sp. are not detectable in bile from German patients with biliary disease. Gastroenterology 1999; **116:** 1016–7.
19. Kalos A, Delidou A, Kordosis T, Archimandritis A, Gaganis A, Angelopoulos B. The incidence of gallstones in Greece; an autopsy study. Acta Hepatogastroenterol (Stuttg) 1977; **24:** 20–3.
20. Archimandritis A, Bitsikas J, Tjivras M, Fertakis A, Anastasakou E, Pitsouni E et al. Helicobacter pylori infection in Greece in healthy people and in patients with peptic ulcer and with dyspepsia without ulcer. J Clin Gastroenterol 1993; **16:** 257–8.
21. Rosai J. Cholelithiasis *In*: Rosai and Acherman's surgical pathology, 9th edition, Mosby; 2004: 1038–39.
22. Hammar M, Tyszkiewicz T, Wadstrom T, O'Toole PW. Rapid detection of Helicobacter pylori in gastric biopsy material by polymerase chain reaction. J Clin Microbiol 1992; **30:** 54–8.
23. Smith SI, Oyedeji KS, Arigbabu AO, Kantet F, Mergaud F, Ojo OO *et al*. Comparison of three PCR methods for detection of Helicobacter pylori DNA and detection of cagA gene in gastric biopsy specimens. World J Gastroenterol 2004; **10:** 1958–60.
24. Neri V, Margiotta M, de Francesco V, Ambrosi A, Della Valle N, Fersini A et al. DNA sequences and proteic antigens of *H pylori* in cholecystic bile and tissue of patients with gallstones. Aliment Pharmacol Ther 2005; **22:** 715–20.
25. Silva CP, Pereira-Lima JC, Oliveira AG, Guerra GB, Marques DL, Sarmanho L et al. Association of the presence of Helicobacter in gallbladder tissue with cholelithiasis and cholecystitis. J Clin Microbiol 2003; **41:** 5615–8.
26. Fox JG, Dewhirst FE, Shen Z, Feng Y, Taylor N, Paster B et al. Hepatic Helicobacter species identified in bile and gallbladder tissue from Chileans with chronic cholecystitis. Gastroenterology 1998; **114:** 755–63.
27. Apostolov E, Al-Soud WA, Nilsson I, Kornilovska I, Usenko V, Lyzogubov V et al. Helicobacter pylori and other Helicobacter species in gallbladder and liver of patients with chronic cholecystitis detected by immunological and molecular methods. Scand J Gastroenterol 2005; **40:** 96–102.
28. Chen W, Li D, Cannan RJ, Stubbs RS: Common presence of Helicobacter DNA in the gallbladder of patients with gallstone diseases and controls. Dig Liver Dis 2003; **35:** 237–43.
29. Nilsson B, Friman S, Thune A, Jivegard L, Svanvik J. Inflammation reduces mucosal secretion of hydrogen ions and impairs concentrating function and luminal acidification in feline gallbladder. Scand J Gastroenterol 1995; **30:** 1021–6.
30. Apostolopoulos P, Vafiadis-Zouboulis I, Tzivras M, Kourtessas D, Katsilambros N, Archimandritis A. Helicobacter pylori *H pylori* infection in Greece: the changing prevalence during a ten-year period and its antigenic profile. BMC Gastroenterol 2002; **2:** 11.