CLINICAL MICROBIOLOGY

Serological and direct diagnosis of *Helicobacter pylori* in gastric carcinoma: a case-control study

DULCIENE M. M. QUEIROZ, EDILBERTO N. MENDES, GIFONE A. ROCHA, ANDREIA M. R. OLIVEIRA, CELSO A. OLIVEIRA, MÔNICA M. D. A. CABRAL*, ANA M. M. F. NOGUEIRA* and ANTÔNIO F. SOUZA†

Laboratory of Research in Bacteriology, *Department of Pathology, Faculdade de Medicina, Universidade Federal de Minas Gerais and †Laboratory of Pathology, Hospital Luxemburgo, Belo Horizonte, Minas Gerais, Brazil

This study evaluated the sensitivity of serological and direct methods for the diagnosis of *Helicobacter pylori* infection in 127 patients with gastric carcinoma and in 127 controls without this disease, matched for age and sex. Antral and oxyntic mucosal specimens were obtained from all patients, at operation in patients with gastric carcinoma and at endoscopy from controls. The urease test, microscopy of stained smears and culture for *H. pylori* were performed on all specimens. Sera from all patients were tested for antibodies to *H. pylori* by a highly sensitive and specific IgG-ELISA. Culture, urease test, stained smear and ELISA were significantly less sensitive in the patients with gastric carcinoma than in control subjects. However, the combination of several methods improved the diagnosis of *H. pylori* infection in the gastric carcinoma group. Infection was significantly more frequent in the gastric carcinoma patients than in the controls. *H. pylori* infection was associated with an increased risk of developing gastric carcinoma.

Introduction

Although the incidence of gastric carcinoma has decreased markedly in developed countries in recent decades, this disease remains one of the most frequent and lethal malignancies worldwide, particularly in developing countries [1, 2].

Gastric carcinoma has long been recognised as the result of a prolonged process related to an abnormal gastric micro-environment. *Helicobacter pylori* is a major cause of human gastritis and is now accepted as one of the most important inducers of the gastric alterations. Unless it is treated with antimicrobial agents, *H. pylori* gastritis persists throughout life and, in some cases, progresses to chronic atrophic gastritis with intestinal metaplasia and epithelial dysplasia, conditions that have long been thought to play a role in the pathogenesis of gastric carcinoma [3]. There is now consistent evidence implicating *H. pylori* as a gastric carcinogen. A working group of the International Agency for Research on Cancer [4] has

concluded that the micro-organism is carcinogenic for man. The strongest evidence in support of this view originated from three nested case-control studies in which serum samples employed for diagnosing *H. pylori* infection had been obtained several years before diagnosis of gastric cancer [5–7] and the resulting meta-analysis [8]. However, there are discrepancies in the results of studies examining the point prevalence of *H. pylori* infection in gastric carcinoma patients [9–13]. In some of them, the rates of *H. pylori* infection were higher in gastric carcinoma patients than in control populations [11, 13], although in others the rate of infection was similar [12, 14] or lower [9, 10] in the former group. This may be due to the fact that gastric atrophy and intestinal metaplasia are frequently present in stomachs with tumours and they reduce the bacterial load [15, 16]. Furthermore, serum levels of IgG against *H. pylori* also seem to be lower in patients with gastric carcinoma.

In a previous study, a high prevalence of *H. pylori* infection was observed in patients with gastric carcinoma, but a control group was not studied, which would have been necessary as the prevalence of *H. pylori* infection is high in the general population of developing countries [17]. Therefore, in the present study, several gastric fragments were obtained for the

Received 23 Jan. 1998; revised version received 4 Aug. 1998; accepted 4 Sept. 1998.

Corresponding author: Professor D. M. M. Queiroz (e-mail: queiroz@medicina.ufmg.br).

diagnosis of *H. pylori* infection by culture, urease test and stained smear. Sera were obtained to detect antibodies against *H. pylori* in a cross-sectional case-control study of the association between *H. pylori* infection and gastric carcinoma risk in a developing Western country. The study also compared the sensitivity of these methods for diagnosing *H. pylori* infection in patients with and without gastric carcinoma.

Patients and methods

This study was approved by the Ethics Committee of the University Hospital, Universidade Federal de Minas Gerais, Brazil. Informed consent to participate was obtained from all patients.

Patients

Between July 1995 and Aug. 1997, 127 consecutive patients with gastric carcinoma (19 of the oesophago-gastric junction or cardia and 108 of the distal stomach) whose tumour was resected were evaluated. Each patient with carcinoma was matched by age (± 1 year) and gender with a control subject. All patients were of low socio-economic status and were born and resident in Minas Gerais state, Brazil.

The controls without gastric carcinoma were selected from consecutive patients who underwent upper gastrointestinal endoscopy for the evaluation of symptoms involving the upper gastrointestinal tract during the same period of study. All patients with peptic ulcer, coagulation disorders, complications such as gastric perforation or haemorrhage, anatomical obstacles preventing endoscopy and patients who had already undergone a surgical operation were excluded from the study. No patient had received antimicrobial drugs, H_2 -receptor antagonists, acid pump inhibitors, non-steroidal anti-inflammatory drugs or any medication for at least 30 days before the study, except five patients with gastric carcinoma who were using H_2 -receptor antagonists before surgery. Fragments of the gastric mucosa were obtained from the control patients by oesophagogastroduodenoscopy. The endoscope (Olympus, Japan), including all channels and biopsy forceps, was carefully cleaned with water and disinfected by immersion in glutaraldehyde 2.0% solution for 20 min, rinsed in water and dried after each use. In patients with gastric carcinoma, the fragments of the gastric mucosa were obtained from the stomach removed by gastrectomy after opening it along the greater curvature within 1 h of resection.

Microbiological study

In the control group, multiple fragments were obtained from the lesser curvature of the antrum and the greater curvature of the corpus. In the carcinoma group

fragments of tissue were obtained from the antrum and body whenever possible and were taken from epithelium of normal microscopic appearance as far from the tumour as possible. The fragments were held in sodium thioglycollate broth (Difco) at 4°C for a maximum of 1 h. Four fragments from the antral mucosa and four from the oxyntic mucosa were ground separately in a tissue homogeniser and plated on to four petri dishes (two for each region) containing freshly prepared Belo Horizonte medium [18]. No plate was >2 days old at the time of use. After seeding, the plates were incubated at 37°C under micro-aerobic conditions produced by a gas generation kit (Anaerocult C, Merck®). The petri dishes were examined after incubation for 3 days. When no growth was observed, the plates were streaked again with a platinum loop, re-incubated and inspected at 3-day intervals. Plates were discarded after incubation for 12 days. The isolates were identified on the basis of macroscopic colonial appearance and microscopic morphology after carbol fuchsin staining [19], by a rapidly positive urease test, and by positive oxidase and catalase reactions.

Two fragments from the antrum and two from the corpus were used for the direct urease test and for carbol fuchsin-stained smears.

To avoid bias, the slides of the smears were coded. Although the operators could not be blinded to the source of the material because the shape of the fragments obtained from gastric carcinoma patients was different from that of fragments obtained by biopsy forceps from control patients, the technicians who performed the urease tests and all cultures of the clinical studies were unaware of these studies. After the primary culture, the appearance of culture plates became similar and the plates were coded to avoid bias.

Serological study

Blood samples were obtained from all patients. After clotting, the sera were obtained by centrifugation, divided into aliquots and stored at -20°C until they were used. The presence of IgG antibodies specific to *H. pylori* was measured by a second generation ELISA (Cobas-core, anti-*H. pylori* EIA, Roche, Switzerland) which had been validated previously for the Brazilian adult population in terms of culture, preformed urease test and stained smear, and had shown 95.4% sensitivity and 100% specificity [20]. The operator who performed the assays was blinded to the identity of the patients from whom the serum was obtained, in accordance with the recommendations of the manufacturer of the kit.

All serum samples from gastric carcinoma patients and controls that gave negative or doubtful results by ELISA, or tested positive when no other test was positive, were re-tested.

Forty-nine serum samples from patients with gastric carcinoma and 30 from patients in the control group were selected at random for the re-assay (intra-assay and inter-assay) to ensure reliability.

Histopathological study

Tumour fragments were fixed in formalin 10%, dehydrated in alcohol and xylene and embedded in paraffin. Sections (5- μ m thick) stained with haematoxylin and eosin were examined by light microscopy by three pathologists who were unaware of the results. The tumours were classified as intestinal type and diffuse type carcinoma according to Laurén [21]. When the tumour had characteristics of both types it was classified as mixed.

Statistical analysis

Matched Odds ratios (OR) with 95% confidence intervals (CI) were estimated to examine the magnitude of the association between *H. pylori* infection and gastric carcinoma. Subgroups were formed by classifying the matched data sets with respect to gender and to diffuse or intestinal types of tumour. The statistical significance of the Odds ratios was also tested by the χ^2 test with Yates's correction or Fisher's test. The differences in the sensitivity of the urease test, carbol fuchsin staining, culture and serology between gastric carcinoma patients and controls were calculated by the two-tailed Fisher's test or by the χ^2 test with Yates's correction. For evaluation of the ELISA sensitivity the results of the first assay were considered, and when they were doubtful, they were considered to be negative. The mean ages were compared by Student's *t* test. The level of significance was set at $p < 0.05$.

Results

The features of the gastric carcinoma patients and their sex- and age-matched controls are shown in Table 1. The mean age of patients with the diffuse type of tumour was significantly lower ($p = 0.001$) than that of patients with the intestinal type. No other difference in demographic data was observed.

Methods employed for the diagnosis of *H. pylori* infection

Patients were considered to be *H. pylori*-positive if two or more methods, whatever their nature, were positive or if only the culture or ELISA test was positive, as no false-positive results are expected for culture and the specificity of the ELISA employed is 100% for our general population [20].

H. pylori infection was detected in 121 patients (95.3%) with gastric carcinoma. In 106 of these patients the infection was observed by at least two methods. In eight patients (6.3%) the infection was detected by serology alone and in seven patients (5.5%) by culture alone. In these culture-positive patients, 3–10 colonies were observed on at least one plate. *H. pylori* infection was not detected by any method in six patients (4.7%). In the control group, *H. pylori* infection was observed in 95 patients (74.8%). Among the control group subjects, the infection was always detected by two or more tests.

H. pylori infection and gastric carcinoma

A significant association was found between *H. pylori* infection and gastric carcinoma ($p = 0.00002$; OR = 6.8, CI 2.6–18.9). The association was also significant when the tumour was stratified into intestinal and diffuse type, in males and females (Table 2). When the data were stratified by localisation of the tumours, *H. pylori*-positive status was linked only to distal tumours, with the association being stronger than that observed for all cancers (OR = 16.8; CI 95% = 3.7–52.5; $p = 0.00000007$). The risk of developing proximal carcinoma was not increased by *H. pylori* infection (OR = 1; CI = 0.1–3.9; $p = 1$), but the small number of subjects in this subgroup makes the risk estimate imprecise. The 108 subjects with distal tumours were also stratified by sex and type of tumour. Significant differences were observed between each subgroup, i.e., intestinal and diffuse histological types, males and females and their matched controls (Table 2).

When the prevalence of the infection was evaluated by each test separately, no association was observed between carcinoma patients and *H. pylori* infection detected by carbol fuchsin ($p = 0.3$), serology ($p = 0.2$), and culture ($p = 0.4$). An inverse association was

Table 1. Characteristics of patients with gastric carcinoma and control subjects

Patient groups	Number (%)	Female/male	Age	
			Mean (SD)	Range
Carcinoma	127 (100)	35/92	61.6 (13.6)	32–96
intestinal	86 (67.7)	21/65	64.6 (12.7)	32–96
diffuse	35 (27.6)	13/22	54.2 (12.8)	33–77
mixed	6 (4.7)	1/5	61.2 (17.8)	32–84
Controls	127 (100)	35/92	61.4 (13.7)	32–96

Table 2. Risk for gastric carcinoma according to *H. pylori* status

Criteria	OR	CI 95%	p value
All gastric carcinoma	6.8	2.6–18.9	0.000002
intestinal type	6.6	2.0–24.0	0.0005
diffuse type	10.1	1.1–62.4	0.03
male	6.5	2.0–23.6	0.0006
female	5.7	1.1–25.5	0.04
Proximal tumour	1.00	0.1–3.9	1
Distal tumour	16.8	3.7–52.5	0.00000007
intestinal type	12.4	2.6–42.9	0.00006
diffuse type	*	...	0.01
male	10.7	2.2–37.8	0.0003
female	*	...	0.002

*OR non-calculable because after the stratification none of the patients with the diffuse type and none of the female patients was *H. pylori* negative.

observed when only urease was considered in the diagnosis (OR = 1.4; CI 95% = 0.3–0.8; $p = 0.004$).

Sensitivity of the methods employed for the diagnosis of H. pylori infection in patients with and without gastric carcinoma

The most sensitive methods in the diagnosis of *H. pylori* infection in carcinoma patients were culture and serology (83.5%). All tests used were significantly more sensitive in the control patients than in carcinoma patients (Table 3). The differences in sensitivity of the tests in the gastric carcinoma patients were not related to age ($p > 0.3$ for all tests) when the patients were stratified into groups (below and above 40, 50 or 60 years), to sex ($p > 0.2$ for all tests) or to histopathological type of tumour ($p > 0.6$ for all tests).

Inter-assay and intra-assay variability of the ELISA

The inter-assay variability of the ELISA gave qualitatively identical results in 29 (96.7%) of 30 serum samples from the control group and in 42 (85.7%) of 49 samples from the carcinoma patients. Also, no qualitative differences were observed when intra-assay variability was studied in the control group. However, four serum samples from carcinoma patients

showed different results (negative and doubtful in one, doubtful and negative in two, and positive and doubtful in one) in the intra-assay evaluation. In all discordant results, the antibody titres were quite close to the cut-off recommended by the manufacturer to discriminate between positive and negative samples.

Discussion

The prevalence of gastric carcinoma has decreased in industrialised Western countries [1]. However, it remains unchanged in developing countries such as Brazil, where it is the second commonest tumour in males and the fourth commonest in females [22].

H. pylori is now considered to be an important factor in the pathogenesis of gastric carcinoma. However, studies examining the point prevalence of *H. pylori* infection in gastric carcinoma patients present divergent results. In many of them, the prevalence of the infection was similar [12, 14] or lower [9, 10] in carcinoma patients than in the control group. These discrepancies may be linked to a low sensitivity of the methods used in the diagnosis of *H. pylori* infection in gastric carcinoma patients caused by changes in the gastric micro-environment that reduce *H. pylori* colonisation or to an age-related increase in the prevalence of infection among control subjects, especially in developing countries where *H. pylori* infection is quite prevalent in the general population.

Thus, in this study, the point prevalence of *H. pylori* infection in patients with gastric carcinoma was evaluated by means of several tests to improve the diagnosis of *H. pylori* infection. The results showed that *H. pylori*-positive individuals have a 16.8-fold higher chance of having distal gastric carcinoma than those not infected with the micro-organism. On the other hand, subjects infected by *H. pylori* had no increased risk of developing proximal tumours. Although the small number of patients in this subgroup makes the risk estimate imprecise, the hypothesis that there is no association between *H. pylori* infection and proximal gastric carcinoma is

Table 3. Sensitivity of the methods employed for diagnosis of *H. pylori* infection in gastric carcinoma patients and in control subjects

Method	Sensitivity (%)		p
	Gastric carcinoma patients	Controls	
Culture	83.5	97.9	0.0004
Urease test	56.2	93.7	$<10^{-7}$
Stained smear	67.8	92.6	0.00002
ELISA	83.5	94.7	0.02

Sensitivity was calculated by dividing the number of each positive test separately by the number of patients that were considered *H. pylori*-positive $\times 100$. The number of patients considered to be *H. pylori*-positive was 121 among carcinoma patients and 95 among control patients.

supported by the fact that the incidence of carcinoma of the proximal stomach has been rising and *H. pylori* infection has been decreasing in developed countries.

In this study, if only one test (culture, carbol fuchsin-stained smear, urease test or ELISA) had been used to make the diagnosis of *H. pylori* infection, no association between distal carcinoma and *H. pylori*-positive status could have been observed, as none of the tests individually presented good sensitivity in gastric carcinoma patients. It has been pointed out that serum antibody detection is more sensitive than direct tests for *H. pylori* diagnosis in gastric carcinoma patients, because antibodies persist after the disappearance of the micro-organism that is supposed to occur in cancerous tissue and in lesions that co-exist with it, such as atrophy and intestinal metaplasia. Taken together, the results of the present study demonstrate that, in fact, the bacterial load was decreased in carcinoma patients, as the sensitivity of culture, urease test and carbol fuchsin-stained smears was lower than in the control group.

Decreased detection of *H. pylori* by direct methods has also been demonstrated in gastric carcinoma patients by others [23]. Although the bacterial count seems to be decreased in patients with gastric carcinoma, the present study found that culture was one of the most sensitive methods of diagnosis of *H. pylori* infection in this group of patients. This finding has no precedent in the literature. In several studies, even when carcinoma patients are not included, culture has not been the most sensitive method for *H. pylori* diagnosis. However, in our experience, when the conditions are optimal, culture is a very sensitive method. Such conditions include a proper micro-aerobic atmosphere, the use of good, recently prepared medium, staff trained in this procedure and rigorous evaluation of the plates at 3-day intervals. Furthermore, several mucosal fragments from different regions of the stomach were used to cultivate *H. pylori*, which may have overcome the possible uneven distribution of the micro-organism in the stomach. Also, areas far from the cancerous tissues were evaluated and these areas are more favourable for the detection of *H. pylori*. Even when these procedures were carefully adopted, few colonies on only one plate or absence of growth after 3 days of incubation were observed with samples from some gastric carcinoma patients; however, growth became abundant after the plates had been streaked again and re-incubated for 3 or 6 more days. It is also worth emphasising that the patients had not received antimicrobial drugs, which may suppress the infection and produce a false-negative culture.

In this study, the sensitivity of the ELISA test was significantly lower in cancer patients than in controls, a fact possibly related to a decreased bacterial load or to the possible modulation of the immune response in

patients with cancer, or to the nature of the serological method used, or a combination of these factors. The last possibility is unlikely, because a highly sensitive and specific ELISA was used, as demonstrated in the control group and in a population previously studied to validate the test [20]. Although some negative serological tests could be explained by the decreased bacterial load, this cannot explain all negative cases. In fact, the ELISA was negative in eight patients in whom a large number of micro-organisms was present, indicating that some patients with gastric carcinoma are unable to respond well to *H. pylori* infection with specific antibodies. Thus, in many studies the detection rate of *H. pylori* infection in gastric carcinoma patients by ELISA alone may give an underestimate. Crabtree *et al.* [24], comparing the results of ELISA and immunoblotting in the diagnosis of *H. pylori* infection in gastric carcinoma patients, also observed that ELISA results underestimate the true estimate.

In conclusion, as individual diagnostic tests are less accurate than a group of tests in determining current *H. pylori* infection in gastric carcinoma patients, on the basis of this study we recommend the association of at least a direct (culture) and an indirect (serology) test. The use of several methods made it possible to demonstrate that *H. pylori* infection is strongly associated with an increased risk for the development of distal gastric carcinoma in a developing Western country.

This work was supported by grants from CNPq, FINEP and FAPEMIG/Brazil. We gratefully acknowledge the surgeons of the University Hospital/UFMG, Hospital Luxemburgo and Hospital Mário Pena who kindly assisted in the collection of the stomach samples.

References

1. Howson CP, Hiyama T, Wynder EL. The decline in gastric cancer: epidemiology of an unplanned triumph. *Epidemiol Rev* 1986; **8**: 1–27.
2. Parkin DM, Läärä E, Muir CS. Estimates of the worldwide frequency of sixteen major cancers in 1980. *Int J Cancer* 1988; **41**: 184–197.
3. Correa P. A human model of gastric carcinogenesis. *Cancer Res* 1988; **48**: 3554–3560.
4. International Agency for Research in Cancer: Schistosomes, Liver Flukes and *Helicobacter pylori*. Lyon: IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol 61. 1994.
5. Forman D, Newell DG, Fullerton F *et al.* Association between infection with *Helicobacter pylori* and risk of gastric cancer: evidence from a prospective investigation. *BMJ* 1991; **302**: 1302–1305.
6. Nomura A, Stemmermann GN, Chyou P-H, Kato I, Pérez-Pérez GI, Blaser MJ. *Helicobacter pylori* infection and gastric carcinoma among Japanese-Americans in Hawaii. *N Engl J Med* 1991; **325**: 1132–1136.
7. Parsonnet J, Friedman GD, Vanderssteen DP *et al.* *Helicobacter pylori* infection and the risk of gastric carcinoma. *N Engl J Med* 1991; **325**: 1127–1131.
8. Forman D, Webb P, Parsonnet J. *H. pylori* and gastric cancer. *Lancet* 1994; **343**: 243–244.
9. Jiang SJ, Liu WZ, Zhang DZ *et al.* *Campylobacter*-like organisms in chronic gastritis, peptic ulcer, and gastric carcinoma. *Scand J Gastroenterol* 1987; **22**: 553–558.

10. Feng Y-Y, Wang Y. *Campylobacter pylori* in patients with gastritis, peptic ulcer, and carcinoma of the stomach in Lanzhou, China. *Lancet* 1988; **1**: 1055–1056.
11. Hansson L-E, Engstrand L, Nyrén O *et al.* *Helicobacter pylori* infection: independent risk indicator of gastric adenocarcinoma. *Gastroenterology* 1993; **105**: 1096–1103.
12. Kuipers EJ, Gracia-Casanova M, Peña AS *et al.* *Helicobacter pylori* serology in patients with gastric carcinoma. *Scand J Gastroenterol* 1993; **28**: 433–437.
13. Hu PJ, Mitchell HM, Li YY, Zhou MH, Hazell SL. Association of *Helicobacter pylori* with gastric cancer and observations on the detection of this bacterium in gastric cancer cases. *Am J Gastroenterol* 1994; **89**: 1806–1810.
14. Loffeld RJF, Willems I, Flendrig JA, Arends JW. *Helicobacter pylori* and gastric carcinoma. *Histopathology* 1990; **17**: 537–541.
15. Karnes WE, Samloff IM, Siurala M *et al.* Positive serum antibody and negative tissue staining for *Helicobacter pylori* in subjects with atrophic body gastritis. *Gastroenterology* 1991; **103**: 167–174.
16. Osawa H, Inoue F, Yoshida Y. Inverse relation of serum *Helicobacter pylori* antibody titres and extent of intestinal metaplasia. *J Clin Pathol* 1996; **49**: 112–115.
17. Nogueira AMMF, Ribeiro GM, Rodrigues AG *et al.* Prevalence of *Helicobacter pylori* in Brazilian patients with gastric carcinoma. *Am J Clin Pathol* 1993; **100**: 236–239.
18. Queiroz DMM, Mendes EN, Rocha GA. Indicator medium for isolation of *Campylobacter pylori*. *J Clin Microbiol* 1987; **25**: 2378–2379.
19. Rocha GA, Queiroz DMM, Mendes EN, Lage AP, Barbosa AJA. Simple carbolfuchsin staining for showing *C. pylori* and other bacteria in gastric mucosa. *J Clin Pathol* 1989; **42**: 1004–1005.
20. Rocha GA, Oliveira AMR, Queiroz DMM *et al.* Serodiagnosis of *H. pylori* infection by Cobas core ELISA in adults from Minas Gerais, Brazil. *Braz J Med Biol Res* 1998; **31**: 1263–1268.
21. Laurén P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. *Acta Pathol Microbiol Scand* 1965; **64**: 31–49.
22. Instituto Nacional de Câncer, Programa de Oncologia: Registro Nacional de Patologia Tumoral, Diagnóstico de Câncer, Brasil 1981–85. Brasília, Ministério da Saúde. 1991: 1–326.
23. Clarkson KS, West KP. Gastric cancer and *Helicobacter pylori* infection. *J Clin Pathol* 1993; **46**: 997–999.
24. Crabtree JE, Wyatt JJ, Sobala GM *et al.* Systemic and mucosal humoral responses to *Helicobacter pylori* in gastric cancer. *Gut* 1993; **34**: 1339–1343.