MIXTURE OF CARBOL FUCHSIN AND ALCIAN BLUE STAINING OF GASTRIC TISSUE FOR THE IDENTIFICATION OF *HELICOBACTER PYLORI* AND GOBLET CELL INTESTINAL METAPLASIA

Sirisanpang Yodavudh[1], Siriwan Tangjitgamol[2] and Supalarp Puangsa-art[3]

[1]Department of Pathology, Chareonkrung Pracharak Hospital, Bangkok; [2]Department of Anatomical Pathology and Department of Obstetrics and Gynecology, Bangkok Metropolitan Administration Medical College and Vajira Hospital, Bangkok; [3]Department of Tropical Hygine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

Abstract. The purpose of this study was to evaluate the role of the mixture of carbol fuchsin and alcian blue stain in the diagnosis of *Helicobacter pylori* (HP) and goblet cell intestinal metaplasia (IM) in comparison to the more commonly used Giemsa and hematoxylin and eosin (H&E) stains. Pathological blocks of gastric tissues obtained from January 2006 to December 2007 were recut and processed for Giemsa and a mixture of carbol fuchsin and alcian blue stains. Clinical data regarding the patients were collected and previous slides stain with H&E from gastric tissues were reviewed.The Giemsa and the mixture of carbol fuchsin and alcian blue stains were studied by a pathologist who was blinded to the pathological and clinical data. Direct comparisons were made between the stains for diagnosis of HP. Of 423 cases studied the concordance rate was 97.8% (kappa value=0.947, $p< 0.05$). Using the mixture of carbol fuchsin and alcian blue stain, 4.3 % of goblet cell IM which were not detected by H&E stain were additionally identified. The prevalences of HP infection diagnosed by Giemsa, the mixture of carbol fuchsin and alcian blue, and H&E stains were 72.1%, 72.3%, and 71%, respectively. In conclusion, the mixture of carbol fuchsin and alcian blue stain can be used in place of Giemsa stain for the identification of HP, and is probably preferable because of its low cost and is less time-consuming. Carbol fuchsin and alcian blue which are commonly available dyes are more beneficial than Giemsa stain and aid in identifing goblet cell IM undiagnosed by conventional H&E stain.

INTRODUCTION

Helicobacter pylori (HP) is an important etiologic organism causing various gastric pathologies, such as acute and chronic gastritis, gastric ulcers, gastric carcinoma, and low grade B-cell gastric (MALT type) lymphoma (Covaccci and Rappuoli, 2003; Blaser and Atherton, 2004). Of these, gastric cancer is probably the most catastrophic sequelae of HP infection. Thus, the detection of HP has become essential in the clinical and pathological investigations of gastric disease.

HP may be detected by simple non-invasive serologic methods of the Clo test and saliva IgA (Kullavanijaya *et al,* 2004) or the more invasive method of gastric mucosa biopsy. The latter is more reliable because the bacterial organism may be directly visualized on histologic examination (Suwanagool *et al,* 1993). Although HP is readily seen on hema-

Correspondence: Dr Sirisanpang Yodavudh, Department of Pathology, Charoenkrung Pracharak Hospital, 8 Charoenkrung Road, Bangkok 10120, Thailand.
Tel: 66 (0) 2289-7774, 08-1842-7313; Fax: 66 (0) 2289-7774
E-mail: sirisanpang@hotmail.com

toxylin and eosin (H&E) stain of gastric biopsy specimens, special stains, such as Warthin-Starry, Giemsa (Garvey *et al,* 1985; Kolts *et al,* 1993), genta stain, toluidine blue stain and immunohistochemical studies (Goodwin *et al,* 1997) have been used for a better identification of HP. Although the diagnostic performances (specificities, sensitivities and accuracies) of each method from these studies varied, no clear advantage of one method over the others has been demonstrated (Toulaymat *et al,* 1999; Jhala *et al,* 2002). At present, Giemsa stain is the most widely used because it is easily performed and may be repeated without excessive cost on subsequent tissue biopsies during follow-up examinations by a gastroenterologist (Suwanagool *et al,* 1993). Some authors propose the modified triple stain using carbol fuchsin, alcian blue and H&E improves the rate of HP detection (EL-Zimaity, 2000).

Aside from being infected with HP, the other predisposing pathologic condition of gastric cancer is goblet cell intestinal metaplasia (IM) (Correa, 1988). Goblet cell IM is diagnosed by the morphologic changes of the gastric mucosa on H&E stain. However, the pathologist may fail to notice and report this pathology. Goblet cell IM may be accentuated by the use of alcian blue staining to identify acid mucin (such as sialomucin and sulfomucin) which is secreted by goblet cells (Reis *et al,* 1999).

Because HP and goblet cell IM play important roles in the gastric carcinoma, any means to increase their detection rates may be useful. Our study aimed to evaluate the role of the mixture of carbol fuchsin and alcian blue stain, which is a simple and inexpensive staining method to improve HP and goblet cell IM detection.

MATERIALS AND METHODS

This study obtained approval from the hospital's ethics committee for research of Bangkok Metropolitan Administration. We searched the archives of the Department of Pathology, Chareonkrung Pracharak Hospital to identify patients who had undergone gastric biopsy at the institution between January 2006 and December 2007. Inclusion criteria were: tissue biopsies from the gastric body or antral mucosa. Exclusion criteria were necrotic gastric tissue, gastric polyps, gastric mucosal epithelial dysplasia, gastric cancer, and inadequate tissue volume to be processed for special staining. Samples of formalin-fixed, paraffin-embedded tissue of patients were identified, retrieved, and processed for Giemsa and mixture of carbol fuchsin and alcian blue staining studies. Clinical data abstracted from the patients' charts included: age, gender, gross clinical features of gastric pathology of gastric ulcer versus non-ulcerative gastritis as has been documented during endoscopic examination.

The mixture of carbol fuchsin and alcian blue staining was performed by immersing the slides in carbol fuchsin and 0.25% hydrogen peroxide for 2 minutes and rinsing with tap water.The slides were then placed in 1% alcian blue and 3% acetic acid at a pH of 2.5 for 1-2 minutes. Slides were rinsed with tap water and dehydrated with absolute alcohol, then cleared in xylene and covered with a coverslip (El-Zimaity, 2000). Total staining time averaged 5 minutes per slide. The slides for each case were labeled with a numerical code and subsequently examined by a pathologist who was blinded to the patient identification and results of the other stains. A positive finding for HP was bright blue staining of the organism by Giemsa as shown in Fig 1 and reddish purple with the mixture of carbol fuchsin and alcian blue staining as displayed in Fig 2. Fig 3 revealed IM lesion by carbol fuschin / alcain blue stain.

Data were analyzed using SPSS statistical software, version 11.5 (SPSS, Chicago, IL).

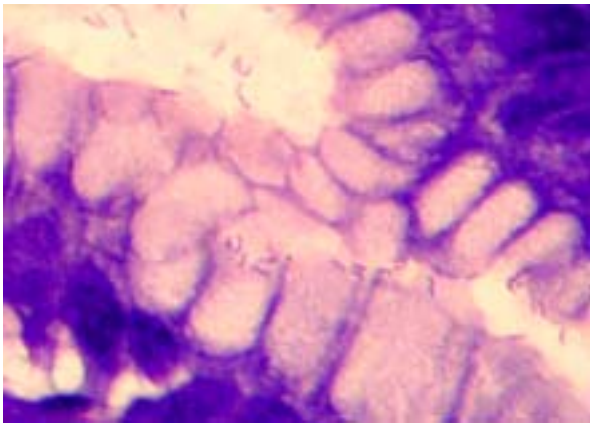

Fig 1–The blue staining of *Helicobactor pylori* by Giemsa stain showing curved bacilli.

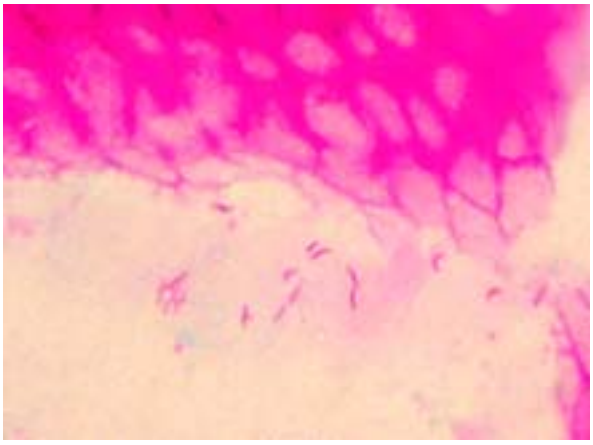

Fig 2–The reddish staining of *Helicobactor pylori* by carbol fuchsin and alcian blue stain revealing curved bacilli.

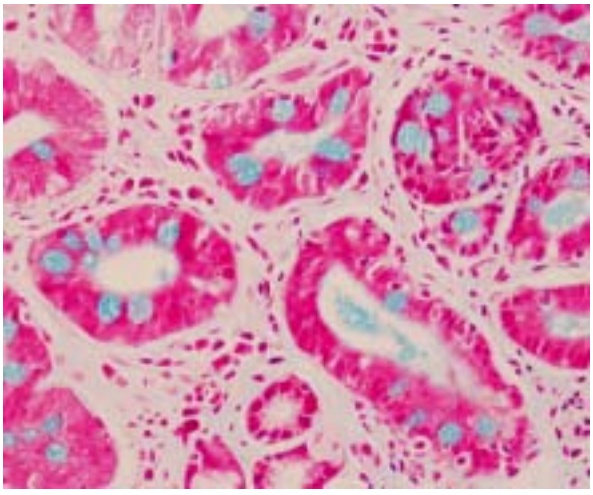

Fig 3–The blue staining of goblet cells by carbol fuchsin and alcian blue stain in complete intestinal metaplasia.

Continuous variables were summarized as means with standard deviation (SD) or medians with range and compared with an unpaired *t*-test. Category variables were reported in numbers and percentages and data from the two groups were compared using the chi - square test.The concordance rate for HP detection between the Giemsa and mixture of carbol fuchsin and alcian blue stain was obtained. Kappa statistics were also performed to assess the agreement between the two stains. Significance was set at $p < 0.05$.

RESULTS

From the archive of the Department of Pathology, 451 gastric tissue specimens were identified during the study period. Twenty-eight cases were excluded: necrotic tissue in 11 cases, polyps in four cases, dysplastic epithelium in 4 cases, cancerous gastric mucosal epithelium in six cases and inadequate tissue for tissue processing in three cases. The remaining 423 cases were included in the study.The mean age of the patients was 56.9±17.3 years (median 57 years, min-max, 15-94 years). More than half the patients were females (225 patients or 53.2%). From the endoscopic medical records, 286 patients (67.6%) were recorded as having nonulcerative gastritis disease (NUD) while the gross lesions in 137 patients (32.4%) were noted as having a gastric ulcer (GU).

Out of 423 biopsied gastric specimens, HP was identified in 305 cases (72.1%) by Giemsa stain and in 306 cases (72.3%) by the mixture of carbol fuchsin and alcian blue stain. The organism was detected in both stains in 301 cases (71.2%) and negative in 113

cases (26.7%). Four hundred fourteen cases had the same diagnosis of either positive or negative stains for HP giving a concordance rate of 97.9% with a kappa value of 0.947 ($p< 0.05$). The comparison of HP identification by the mixture of carbol fuchsin and alcian blue stain versus Giemsa stain are shown in Table 1.

In our study, histomorphologic changes of IM were found on H&E stain in 81/423 cases (19.1%). From the mixture of carbol fuchsin and alcian blue stain, the goblet cells of IM were found in 99 cases (23.4%).The goblet cells of IM were detected in the mixture of carbol fuchsin and alcian blue stain (after being missed by the H&E stain) in 18 cases (4.3%).

We studied the association between HP infection and the clinical features of age, gender, gastroscopic findings, and histomorphologic changes of goblet cell IM. We found no associations between HP infection and age, gender of the patients, or IM. Only gastroscopic findings regarding the features of gastric gross pathology were found to be associated with HP infection. A higher rate of HP infection was found in those with gastric ulcers than in those with non-ulcerative gastritis (Table 2). We also evaluated the association of age and HP findings in patients with gastric ulcers and non-ulcerative (Table 3). No significant differences in mean age were found between HP-positive and HP-negative PU and NUD patients.

The prevalence of HP infection was more common in the 40-80 years old age group. Slight differences in the peak age of prevalence were observed between the groups.In the gastric ulcer group, HP infection was more common in the seventh decade and in the non-ulcer gastritis groups was more common in the sixth decade (Fig 4 and Fig 5).

DISCUSSION

HP is an important cause of gastric pathology. Aside from gastritis and gastric ulcer, another important sequela of this infection is gastric cancer. These conditions make the diagnosis of HP infection important.

The prevalences of HP infection in our study by H&E, Giemsa, and the mixture of carbol fuchsin and alcian blue stains were 71%, 72.1%, and 72.3%, respectively. Our prevalence of 72% was higher than previous studies, which ranged from 37-69% (Kang, 1985; Kang *et al*, 1990; Kanchintorn *et al,* (1992). One study in Thailand by Kanchintorn *et al* (1992) found an HP infection rate of 63.9%. The prevalences of HP infection in our study and in the study by Kanchintorn *et al* (1992) were higher than one report from Singapore (Kang *et al*, 1990).The Singaporean study found HP infection in 59% of 1,502 Singaporean patients undergoing gastroduodenal biopsy most common among Indians (69%), Chinese (60%) and (37%) in Malays. The reasons for these racial

Table 1
Helicobacter pylori identification using carbol fuchsin and alcian blue stain versus Giemsa stain (*N*=423).

		Mixture of carbol fuchsin and alcian blue stain	
		Positive Number (%)	Negative Number (%)
Giemsa stain	Positive	301 (71.2)	4 (0.9)
	Negative	5 (1.2)	113 (26.7)

Table 2
Characteristics of HP positive and negative cases (*N*=423).

Characteristics	HP positive *N*=306 cases Number (%)	HP negative *N*=117 cases Number (%)	p-value
Age (median = 57)			
< 57 (204)	151 (74)	53 (26)	0.456
≥ 57 (219)	155 (70.8)	64 (29.2)	
Sex			
Male (198)	144 (72.7)	54 (27.3)	0.867
Female (225)	162 (72)	63 (28)	
Gastroscopic findings			
Non-ulcer gastritis (286)	195 (68.2)	91 (31.8)	0.006
Gastric ulcer (137)	111 (81)	26 (19)	
Histomorphology of intestinal metaplasia			
Absence (342)	242 (70.8)	100 (29.2)	0.135
Presence (81)	64 (79)	17 (21)	
Total number (423)	306 (72.4)	117 (27.6)	

Table 3
Association between age in HP positive and HP negative results using the mixture of carbol fuchsin and alcian blue stain in peptic ulcer and non-ulcer gastritis patients.

	Number	Mean ± SD	p-value
Age in peptic ulcer patients (years)			
HP positive	111	59.7 ± 15.3	0.160
HP negative	26	64.4 ± 15.8	
Age in non-ulcer gastritis disease (years)			
HP positive	195	54.8 ± 18.0	0.685
HP negative	91	55.8 ± 17.5	

differences remain unclear but environmental factors may play an important role (Kang, 1985). The patient sampling criteria may have varied from study to study.

The age group with the greatest number of HP infections was the 40-80 year old group. A possible explanation for this finding is that there is either reinfection or exposure to different kinds of food in older ages. When we studied the association between various factors and HP infection, we found no statistically significant association between HP infection and age or gender in the presence and absence of IM. Only gastric ulcers had a more significant association with HP infection than non-ulcer gastritis. Our results are similar to those reported by Kang *et al* (1990) who found that duodenal ulcers were more likely to be associated with HP infection than non-ulcer lesions. This is contrary to results of Kachintorn *et al* (1992) who found no significant difference in HP infection between patients with peptic ulcers and non-ulcer gastritis. The conflicting results from these studies

may be explained by the spectrum of diseases and pathologies. In HP infection, peptic ulcers and non-ulcer gastritis are associated with antral gastritis. It is possible that HP causes an opportunistic infection of the gastric mucosa where there is gastritis (Piper, 1985).

Histopathologic changes associated with HP gastritis may vary in severity from mild to marked inflammation with acute or chronic inflammatory cellular infiltration. HP colonizes gastric mucosa in a variety of ways: lying free on the gastric mucus, adhering to surface epithelium, or lodging intercellularly (Chan *et al,* 1992). Factors which influence HP identification are density of infection and experience of the pathologist. Anim *et al* (2000) concluded that H&E stain is adequate for initial assessment. When HP organisms are abundant, they are easily identified by H&E stain. The accuracy of detection of HP by H&E stain (when the lesions have a high density of organisms) is reported to be as high as with other special stains such as Giemsa or genta, 98% compared to 96% and 97%, respectively. However, in cases where there is only mild inflammation, the organism may be difficult to identify on H&E stain; in this scenario other special stains may be helpful (Laine *et al,* 1997). Giemsa stain is easier to perform than Warthin -Starry stain and is commonly used in many laboratories. The mixture of carbol fuchsin and alcian blue stain is less time-consuming (about 5 minutes per slide) compared to Giemsa stain which is about 15 minutes per slide (Kacar *et al,* 2004)

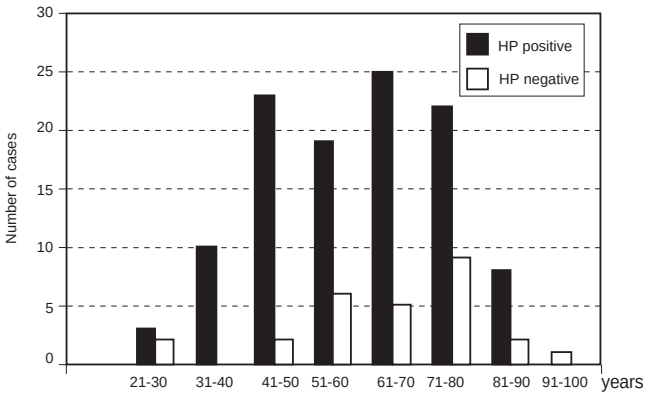

Fig 4–Comparison of age prevalence between HP positive results using the mixture of carbol fuchsin and alcian blue stain (*n*= 111) and HP negative (*n* = 26) patients in the peptic ulcer group (PU).

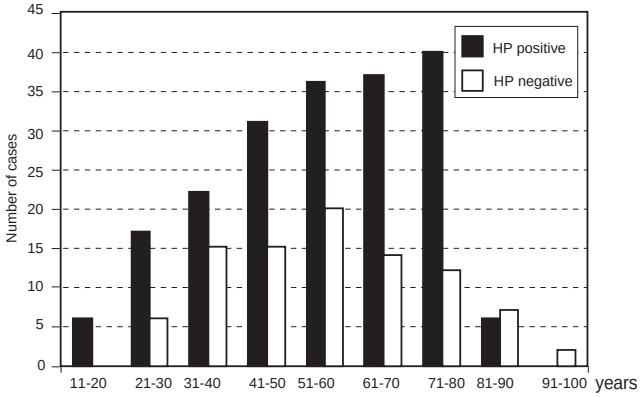

Fig 5–Comparison of age prevalence between HP positive results using the mixture of carbol fuchsin and alcian blue stain (*n*= 195) and HP negative (*n* = 91) patients in the non-ulcer dyspepsia group (NUD).

The detection rates for HP using the mixture of carbol fuchsin and alcian blue and using Giemsa stain were both 71.2%. Concordant diagnoses were found in 414 cases; only nine cases were discordant. The kappa value was 0.947, which is much greater than 0 .75, representing excellent agreement beyond chance (Fleiss, 1981). The 18 cases of goblet cell IM in our study (4.3%), which had been missed on H&E stain, were additionally detected. The false-negative findings on H&E stain could be due to many factors, such as small foci of goblet cell metaplasia, suboptimum quality of the H&E stain, or limited experience of the pathologist in evaluating the histomorphologic changes of intestinal metaplasia. The difference was a modest 4.3% increased rate of detection. Our figure was higher than that reported in a previous study

which found a difference in only 3 cases by alcian blue stain out of 498 gastric biopsies (0.6%) (Wright and Kelly, 2006). Our findings should be confirmed by other studies in order to persuade pathologists to consider alcian blue stain to identify goblet cell IM as an additional pathology and be noted as another risk factor for the patient.

In summary, the mixture of carbol fuchsin and alcian blue stain shows good concordance with Giemsa stain for identifying HP and is probably preferable due to its low cost, its rapidity of performing and its benefit over Giemsa stain in identifing goblet cell intestinal metaplasia.

ACKNOWLEGEMENTS

The authors gratefully acknowledge Mrs Pranee Seangngeon and Mrs Boonruan Pantang for their archive retrieval and staining technical assistance.

REFERENCES

Anim JT, Al-Sobkie N, Prasad A, John B, Sharma PN, Al-Hamar I. Assessment of different methods for staining *Helicobacter pylori* in endoscopic gastric biopsies. *Acta Histochem* 2000; 102: 129-37.

Blaser MJ, Atherton JC. *Helicobactor pylori* persistence: biology and disease. *J Clin Invest* 2004; 113: 321-2.

Chan WY, Hui PK, Leung KM, Thomas TM. Modes of *Helicobacter* colonization and gastroepithelial damage. *Histopathology* 1992; 21: 521-8.

Correa P. A human model of gastric carcinogenesis. *Cancer Res* 1988; 48: 3554-60.

Covacci A, Rappuoli R. *Helicobacter pylori* : after the genomes, back to biology. *J Exp Med* 2003; 197: 807-8.

EL-Zimaity HM. Modified triple stain (Carbol fuchsin / alcian blue / hematoxylin – eosin) for the identification of *Helicobactor pylori*. *Arch Pathol Lab Med* 2000; 124: 1416-7.

Fleiss JL. The measurement of interrater agreement. In: Fleiss JL. Statistical methods for rate and proportions. Philadelphia: John Wiley & Sons, 1981: 127.

Garvey W, Fathi A, Bigelow F. Modified steiner for the demonstration of spirochetes. *J Histotechnol* 1985; 8: 15-7.

Goodwin CS, Mendail MM, Northfield TC. *Helicobacter pylori* infection. *Lancet* 1997; 349: 265-9.

Jhala N, Lechago S, Lechago J, Younes M. Is immunostaining for *Helicobacter pylori* superior to the special stain thiazine in detecting small numbers of *H.pylori* in gastric biopsies? *Appl Immunohistochem Mol Morphol* 2002; 10: 82-4.

Kacar F, Çulhaci N, Yükselen V, Meteoglu I, Dikicioglu E, Levi E. Histologic demonstration of *Helicobacter pylori* in gastric biopsies: Which is the best staining method? *Internet J Pathol* 2004; 3 (1). [Online]. [Cited 2008 Mar 13]. Available from : URL: http://www.ispub.com/ostia/index.php?xmlFilePath =journals/ijpa/vol3n1/pylori.xml

Kachintorn U, Atisook K, Tanwandee T, *et al. Helicobacter pylori* and peptic ulcer disease: Prevalence and association with antral gastritis in 210 patients. *J Med Assoc Thai* 1992; 75: 386-91.

Kang JY, Wee A, Math MV, *et al*. *Helicobacter pylori* and gastritis in patients with peptic ulcer and non - ulcer dyspepsia: ethnic differences in Singapore. *Gut* 1990; 31: 850-3.

Kang JY. Peptic ulcer surgery in Singapore 1951-80 with particular reference to racial differences in incidence. *Aust NZ J Med* 1985; 15: 604-8.

Kolts BE, Joseph B, Achem SR, Bianchi T, Monteiro C. *Helicobacter pylori* detection. A quality and cost analysis. *Am J Gastroenterol* 1993; 88: 650-5.

Kullavanijaya P, Thong-Ngam D, Hanvivatvong O, Nunthapisud P, Tangkijvanich P, Suwanagool P. Analysis of eight different methods for the detection of *Helicobacter pylori* infection in patients with dyspepsia. *J Gastroenterol Hepatol* 2004; 19: 1392-6.

Laine L, Lewin DN, Naritoku W, Cohen H. Prospective comparison of H&E, giemsa, and genta stains for the diagnosis of *Helicobacter pylori. Gastrointest Endosc* 1997; 45: 463 -7.

Piper DW. Bacteria, gastritis, acid hypersecretion and peptic ulcer. *Med J Aust* 1985; 142 : 431.

Reis CA, David L, Correa P, *et al.* Intestinal metaplasia of human stomach displays distinct patterns of mucin (MUC1, MUC2, MUC5AC, MUC6) expression. *Cancer Res* 1999; 59: 1003-7.

Suwanagool P, Atisook K, Pongpech P, Dhiraputra C, Luengrojanakul P, Kachintorn U. *Helicobacter pylori*: a comparison of CLO test and Giemsa's stain with culture in dyspeptic patient. *J Med Assoc Thai* 1993; 76: 185-9.

Toulaymat M, Marconi S, Garb J, Otis C, Nash S. Endoscopic biopsy pathology of *Helicobacter pylori* gastritis. Comparison of bacterial detection by immunohistochemistry and Genta stain. *Arch Pathol Lab Med* 1999; 123: 778-81.

Wright CL, Kelly JK. The use of routine special stains for upper gastrointestinal biopsies. *Am J Surg Pathol* 2006; 30: 357-61.