Technical report

A reliable method for the simultaneous identification of *H pylori* and gastric metaplasia in the duodenum

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Abstract

While an association of *Helicobacter pylori* infection with duodenal mucosa gastric metaplasia has been described, the details and extent of the interaction are lacking. One of the limiting factors has been the lack of a staining technique that allows simultaneous visualisation of the bacteria and gastric metaplasia in the duodenum. This report describes a new stain that allows the simultaneous visualisation of duodenal gastric metaplasia and *H pylori* and compares the new stain with the component stains.

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Previous studies on duodenal gastric metaplasia¹⁻⁵ have been hampered by the lack of a stain that allowed the simultaneous visualisation of *H pylori* and the metaplastic tissue. Previous investigators have been required to use two sets of slides and thus could not directly investigate the possible interactions of the bacteria and the duodenal mucosa. We developed a simple dual stain in which the characteristic morphology of the bacteria and areas with gastric metaplasia are easily visualised.

Methods

HISTOCHEMISTRY

Duodenal biopsy specimen were fixed in 10% buffered formalin, processed, oriented on edge, embedded in paraffin, and cut in sequential 4 µm sections.

STAINING

Four 4 µm sections were cut from each block and stained with haematoxylin and eosin (H&E), Steiner, PAS, and the currently described stain, respectively. Two sets were thus prepared, one consisting of H&E, PAS, and Steiner, and the second, the currently described stain. Each set of slides was separately randomised and coded.

REAGENTS/SOLUTIONS

For the PAS stain

1. 0.5% Periodic acid solution: periodic acid 0.5 g, distilled water 100 ml

2. Schiff's reagent: obtained from Polyscientific (Bayshore, New York, USA)

For silver impregnation

1. 2.5% Gum mastic, 2.5 g: obtained from Polyscientific
2. Lead nitrate-gum mastic solution: Lead nitrate 0.5 g, 70% alcohol 40 ml, Gum mastic (2.5% alcohol) 10 ml (this solution may be reused but should be discarded after two months.)
3. 1% Silver nitrate: silver nitrate 0.5 g, distilled water 50 ml
Make fresh each time and filter before use.
4. 0.04% Silver nitrate: silver nitrate 0.04 g, distilled water 100 ml
Make fresh each time.
5. 2% Hydroquinone: hydroquinone 5 g, distilled water 250 ml
Make fresh each time.
6. Reducing solution: Gum mastic, 2.5% solution, 100 ml hydroquinone, 2% solution, 250 ml absolute alcohol 50 ml
Make just before each use, filter through Whatman No 4 filter paper, and add 25 ml of 0.04% silver nitrate. Do not filter after adding the silver nitrate. This solution will have a milky appearance when the gum mastic is added.

PROCEDURE

Before staining, place a plastic Coplin jar in a 45-50°C water bath to heat. Prepare the reducing solution and place in the preheated Coplin jar.

Staining procedure

1. Deparaffinise and hydrate to distilled water.
2. Oxidise in periodic acid solution for five minutes.
3. Rinse in four changes of distilled water.
4. Place in Schiff's reagent for 6-9 minutes.
5. Wash in running tap water for 10 minutes.
6. Rinse in two changes of distilled water.
7. Sensitise sections by placing in room temperature lead nitrate-gum mastic solution and microwave at high power for 30 seconds.

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8. Allow slides to remain in solution for another 30 seconds (gently stir with a glass rod).
9. Rinse in distilled water.
10. Place sections in 1% silver nitrate, microwave, but do not boil (2 minutes 15 seconds).
11. Allow slides to remain in the warm solution for 90 seconds.
12. Wash in distilled water ($\times 4$).
13. Place in a reducing solution and microwave at high power for 30 seconds.
14. Allow slides to remain in the warm solution for three minutes or until properly developed.
15. Rinse in distilled water ($\times 4$).
16. Hydrate back to distilled water.
17. Stain in Gill's haematoxylin for one minute.
18. Rinse in distilled water.
19. Dehydrate slowly through fresh alcohols. Clear in xylene.
20. Mount with Permount.

EVALUATION OF SENSITIVITY

Selection of biopsy specimens

Large cup forceps duodenal biopsy specimens were chosen from patients with documented *H pylori* infection. The true *H pylori* status was established by consistency of the combination of rapid urease test, culture, and multiple previous and synchronous biopsies of the corpus and antrum for histology. All tests had to be negative to exclude active (uncured) *H pylori* infection. Cases were chosen to ensure approximately equal numbers of cases to represent the full spectrum of duodenal gastric metaplasia (scale 0 to 5).

Examination of the slides

Slides were examined independently by two of us (HE-Z, TA). Observers had no information on the clinical status of the patients and no knowledge of their own or their colleagues' interpretations of the other sets of slides.

A visual analogue scale graded from 0 (absent) to 5 (maximum intensity) was used to score gastric metaplasia and *H pylori*.⁶ A score of 1 indicates that only one or two bacteria were observed on the entire area examined, and a score of 5 indicates that the surface was covered with bacteria and bacterial aggregates. As no stain can be considered the gold standard for identifying *H pylori*, a biopsy specimen was considered positive when bacteria with the characteristic morphological features of *H pylori* were present in any of the slides prepared from the specimen. After results were analysed, all slides that had been erroneously diagnosed with respect to *H pylori* presence or absence by one or more observers were re-examined jointly and a consensus was reached on the bacterial density score. In addition, one pathologist (HE-Z) recorded the time required to examine each slide set. The statistical difference between the two sets was analysed using the χ^2 test.

Results

Gastric mucin had a rose to purple reaction. *Helicobacter pylori* were dark brown to black

Figure 1 (A) Duodenal epithelium with a large segment of gastric metaplastic tissue. Few *H pylori* bacteria are seen (dual stain, original magnification $\times 100$). (B) Duodenal epithelium showing two cells with gastric metaplasia. *H pylori* are seen both next to goblet cells and duodenal gastric metaplasia (dual stain, original magnification $\times 100$).

with darker dot at each pole (fig 1). The order of staining was important because if the silver impregnation method preceded the PAS stain the bacteria did not stain. Lamina propria inflammation can be observed with Gill's haematoxylin. If an H&E stain is desired, it can be included after step 14.

EVALUATION OF SENSITIVITY

We included 109 duodenal mucosal biopsy specimens from 68 patients in the comparative study. Sixty two had gastric metaplasia and 27 had *H pylori* in their duodenal biopsies.

Steiner stain

The Steiner stain detected *H pylori* in 19 of 27 (70%) *H pylori* positive duodenal slides. All false negative results were from slides in which gastric metaplasia or *H pylori* infection had a score of 1 or 2. The sensitivity, specificity, negative predictive value, and positive predictive value for the Steiner stain in this study were 70%, 100%, 91%, and 100%, respectively. The time to examine the slide set was two hours.

PAS/Steiner

H pylori were identified in 26 of 27 (96%) *H pylori* positive slides. There were no false positive readings. As with the Steiner stain, the false negative result was from a slide with a *H pylori* score of 2. The sensitivity, specificity, negative predictive value, and positive predictive value for this stain were 96%, 100%, 99%, and 100%, respectively. The time to examine the slide set was one hour and 30 minutes.

Haematoxylin and eosin and PAS

As expected, H&E was significantly less accurate than either the PAS or the PAS/

Steiner stain ($p < 0.02$) for identifying gastric metaplasia: 34 of 62 positive slides (55%) .

Discussion

H pylori can be difficult to visualise in the duodenum. The new dual stain is a combination of periodic acid-Schiff⁷ and a modified Steiner silver impregnation method.⁸⁻⁹ The correlation between *H pylori* and duodenal gastric metaplasia area was well illustrated without the need to examine two sections. This is especially important because areas of gastric metaplasia are often small and difficult to find, even on sequential slides. The new stain uses lead nitrate rather than uranyl nitrate to avoid radioactivity restriction.¹⁰ We are currently using this stain in our laboratory for the evaluation of duodenal biopsies.

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