HpSS: a new silver staining method for Helicobacter pylori

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Abstract

**Aims**—To verify whether the proposed new silver staining method compares favourably with other well established methods in the detection of Helicobacter pylori in gastric biopsies.

**Methods**—One hundred and forty pairs of antral and fundic biopsies, routinely formalin fixed and paraffin wax embedded, from 70 consecutive unselected patients were stained with haematoxylin and eosin, modified Giemsa, and the proposed H pylori silver stain (HpSS). H pylori immunodetection was performed in the same material with a polyclonal antiserum against H pylori.

**Results**—H pylori was detected in 89 biopsies from 48 patients with haematoxylin and eosin; in a further five biopsies (one antral and four fundic) with Giemsa stain, thereby identifying one more H pylori infected patient. The new silver staining method was positive in all the cases detected by these two methods and detected three extra infected patients (five more positive biopsies). Immunohistochemistry detected one more positive case (two positive biopsies) not identified by any of the other methods.

**Conclusions**—The HpSS method proposed is highly sensitive in detecting H pylori; it is simple and it compares well with other methods used routinely for evaluating gastric biopsies for H pylori.

Keywords: Helicobacter pylori; AgNOR; histochemistry

The discovery of the role of Helicobacter pylori has revolutionised our understanding, diagnosis, and treatment of gastroduodenal diseases. Identification of H pylori in gastric biopsies remains, at present, the most diffuse and accessible method to diagnose H pylori infection.

In most cases, H pylori can be recognised using the haematoxylin and eosin stain. However, specialised stains are necessary when bacteria are rare or when histological sections show chronic active inflammation but no bacteria are identified; in particular, special efforts to identify H pylori are mandatory in control biopsies after antibiotic treatment and in the follow up of patients with gastric lymphoma. Several staining methods for H pylori have been proposed: a modified Giemsa stain and the Warthin-Starry stain are widely used but are of limited sensitivity (Giemsa) or lengthy and capricious (Warthin-Starry). More recently, Genta et al proposed a panoptic stain, which achieves the simultaneous sensitive and clear staining of H pylori and the morphological evaluation of tissue features. However, the first step in this modified Steiner’s stain relies on a toxic and radioactive compound, uranyl nitrate, which is not easily available in many European countries for safety reasons, thus hampering its widespread use. Immunostaining with anti-H pylori antiserum is a highly sensitive and certainly more specific method for identification of H pylori. It is currently considered the gold standard morphological method when an appropriate antigen retrieval protocol is applied; it is especially useful when dealing with coccoid forms but is a costly technique and is not available in all laboratories.

We propose a simple new silver staining method that clearly delineates H pylori and, when coupled with the routine haematoxylin and eosin staining, allows the simultaneous assessment of morphology. We compared the sensitivity of this new method with haematoxylin and eosin, Giemsa, and H pylori immunostaining in a series of gastric biopsies.

**Material and methods**

Routinely formalin fixed and paraffin embedded pairs of antral and corpofundic biopsies (n = 140) from 70 consecutive, unselected patients were evaluated. Histological slides were stained with haematoxylin and eosin, modified Giemsa, the new H pylori silver stain (HpSS), and were immunostained with an anti-H pylori antiserum.

In addition, the above methods were applied to archival gastric biopsies of two patients with Helicobacter heilmannii (formerly Gastrospirillum hominis) infection.

**HELICOBACTER PYLORI SILVER STAINING METHOD**

The HpSS method is summarised in table 1. Deparaffinised and rehydrated sections were rinsed in distilled water and immersed in a freshly prepared 1% AgNO₃ solution. They were transferred to a microwave oven and irradiated at 500 W and at 160 W for 3.3 and 2 minutes, respectively; the solution was not allowed to boil. Slides were rinsed with hot distilled water, briefly left to cool, and then the silver stain for nuclear organiser regions (AgNOR) solution was dropped on to the slides and allowed to react for 30 minutes in the dark. The AgNOR solution was prepared by mixing one part of solution A (2 g of gelatin in
Silver staining solution

A freshly prepared 1% AgNO₃ solution. The AgNOR reaction time must be adjusted in each laboratory as it may vary depending on the temperature and the length of fixation of the specimen. After rinsing in distilled water, slides were immersed in 5% sodium thiosulphate for two minutes and then cleared with blue toning solution (30 mM FeCl₃, 11 mM potassium hexacyanoferrate (III), 33 mM oxalic acid) for 20–30 seconds (blue toning solution remains stable for several months at room temperature). After washing in tap water, the slides were stained with routine haematoxylin and eosin.

**Discussion**

We describe a new simple silver stain that permits an easier identification of *H pylori* in histological sections, allowing a simultaneous and complete assessment of the morphological features associated with *H pylori* gastritis. HpSS allowed the detection of *H pylori* in 74% of the patients in our series, with a 98% sensitivity compared to immunohistochemistry, which can be considered the reference method for *H pylori* identification in gastric biopsies. HpSS proved to be much more sensitive than haematoxylin and eosin staining and even more sensitive than the commonly used Giemsa stain. The high percentage of *H pylori* infected patients in our study reflects the high infection rate of the population under study, in which an elevated incidence of gastric lymphoma and carcinoma has been reported. HpSS is simple and does not require special precautions. When followed by haematoxylin and eosin staining it enables both the sensitive detection of *H pylori* and an adequate evaluation of pathological processes associated with *H pylori* infection to be carried out on one slide. The recently described Genta's stain also couples a silver stain with a routine stain with the aim of: “allowing the simultaneous evaluation on a single slide of bacteriologic and morphologic aspects of *H pylori* associated gastritis.” This stain produces an intense and easy to interpret results, but the initial step requires a solution of uranyl nitrate that is not easily available in several European countries because of radioactive restrictions. For this reason, Genta's stain has not gained popularity in Europe. These problems could be overcome by the use of HpSS, in which all reagents are readily available and simple to prepare. Staining results are easy
New silver staining method for Helicobacter pylori to interpret, well delineating the curved, seagull-shaped morphology of *H pylori* and giving a sharp contrast between bacteria and the epithelial surface or luminal debris, that frequently causes interpretative problems with Giemsa or Warthin-Starry methods. Immunohistochemical detection of *H pylori*, with an adequate pretreatment protocol, is at present the most sensitive and specific among the methods that permit a direct identification of the bacterium; the high sensitivity of this method was demonstrated in this series. However, *H pylori* immunohistochemistry, as well as another potentially highly sensitive and specific morphological method—in situ hybridisation for *H pylori*—are not widely used, and most histopathological laboratories rely on histochemical methods for *H pylori* detection on a routine basis. This new histochemical method could be used in conjunction with haematoxylin and eosin staining in the evaluation of gastri biopsies when the number of bacteria are

Figure 1  Helicobacter pylori associated gastritis: bacteria are stained brown-black by HpSS; original magnification (A) ×400, (B) ×1000

Figure 2  Gastric mucosa infected by Helicobacter heilmannii. (A) HpSS stain, note the polar flagella; (B) the same immunostained with anti- *H pylori* antiserum (original magnification ×1000)
scanty, in particular, after treatment with proton pump inhibitors or unsuccessful eradication therapy. Successful *H pylori* eradication therapy is followed by the rapid and permanent disappearance of neutrophils from the gastric mucosa; the persistence of neutrophil infiltration, albeit focal, should alert the pathologist to the need for a careful search for the bacterium. In cases such as this, a sensitive method like HpSS would be especially useful.

The biochemical basis of the HpSS reaction are obscure. It is known that carboxyl and sulphur-containing groups are of great importance in the AgNOR reaction and probably act as reactive sites for an initial silver deposition that is followed by nucleation of further silver salts. It could be hypothesised that the initial AgNO₃ pretreatment confers a NOR-like reactivity to a particular *H pylori* protein. This reactivity is shared by other bacteria, as demonstrated by a similar reaction with *H heilmanni* and by other non-*H pylori* bacteria sometimes observed in the gastric lumina; it remains to be investigated whether this reaction is restricted to a limited number of bacteria species or is a common property of many bacteria.

Other, non-invasive methods are now available for the diagnosis of *H pylori* infection, such as the urea breath test and serology; their use has been advocated as the methods of choice in evaluating patients with dyspeptic symptoms, avoiding the need for endoscopy. However, only the practice of endoscopy with gastric biopsies will permit a diagnosis of *H pylori* associated diseases, avoiding the risk of missing or delaying the diagnosis of gastric malignancy: histological evaluation of gastric biopsies with a careful search for *H pylori* will remain a significant part of the pathologist's routine work.

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