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Original Research Article

Appraisal of a New Histological Staining Method of *Helicobacter pylori* with *Rubia cordifolia*

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Abstract: ***Background***: Among different histological staining methods for *Helicobacter pylori*, the Haematoxylin and Eosin (H&E) staining method is commonly used in most clinical laboratories. *Rubia cordifolia*, a herbaceous climber, commonly known as the Madder plant is well known for its colour which is commercially exploited in the textile industry. In this study, we explored whether this colouring agent can be utilized for staining *H. pylori* in histological sections. ***Methods***: At first, we studied the nature and intensity of the Rubia root extract with different solvents and mordants. Then we observed that an excellent orange-yellow colour was developed when acetic acid was mixed with ethanol extract of the root powder. ***Results***: A combined Haematoxylin and this extract not only stained *H. pylori* in an excellent way, but also revealed hidden bacteria in gastric pits and mucus very easily. ***Conclusion***: A combined haemaxylin and Rubia (H&R) stain appear ideal for the demonstration of *H. pylori* in histological sections.

Keywords: *Helicobacter pylori*, *Rubia cordifolia*, Haematoxylin and Eosin staining.

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INTRODUCTION

Helicobacter pylori is a gram-negative, spiral-shaped bacterium discovered by Marshall and Warren in Australia in 1983. The microbe is well known to produce gastritis and ulcers. The Cag A toxin-producing strain is now well known as a class I bacterial carcinogen [1] causing gastric carcinoma and gastric mucosa-associated lymphoma. In India, by the adolescent period, about 80 percent population are exposed to the microorganism [2]. There are various methods for demonstration of *H. pylori* in the stomach biopsy samples – culture, H & E staining (haematoxylin and eosin), Gram stain, Giemsa stain, silver stain, immunohistochemistry and rapid urease test (RUT) [1, 9]. Considering the sensitivity, specificity, available infrastructure, cost, and laboratory personnel's choice, H & E staining appears best. Some other staining methods like Wright-Giemsa, thiazine, toluidine blue, and Alcian yellow are also not better than the H & E staining method.

The perennial herbaceous climber *Rubia cordifolia* commonly known as Madder has been used as an eco-friendly natural dye in the textile industry for a pretty long period. The colour of its root extract is due to purpurin (CI-75410) and munjistin (CI-75370), both are anthraquinone derivative glucosides. Besides these two important colouring agents the root also contains small amounts of pseudo purpurin (CI-75420), xanthopurpurin (CI-75340), rubiadin, and nordamncanthal. Madder dye has been used from time immemorial and with different mordants the colouring potential has also been markedly improved. Indian madder is also used for its therapeutic qualities. Its roots are particularly important for its bioactive substances, including anthraquinones. The reddish-brown powdered roots of *R. cordifolia* have a fine, somewhat grainy texture, a bitter flavour, and an earthy smell [10].

The objective of this study is to observe whether *R. cordifolia* root extract can stain *H. pylori* in an optimum way in gastric biopsy tissue sections.

MATERIALS AND METHODS

Collection of Samples

Positive histopathological tissue slides containing *H. pylori* were obtained from the Histopathology Laboratory of a tertiary care hospital in Kolkata, India, following the Institutional Ethics

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Committee guidelines keeping the patients' identity anonymous. In this study, sections from the stored blocks of histologically diagnosed cases as chronic active gastritis with confirmed evidence of *H. Pylori* were considered. A positive result was considered when short, curved, or spiral bacilli were found on the epithelial surface, in the mucus layer, or in gastric pits.

Slide Preparation

The slides were prepared in the histopathology lab using formalin-fixed, paraffin-embedded (FFPE) tissue sections.

Storage and Transportation

The collected slides were stored at room temperature in slide boxes. They were transported to the research facility under controlled conditions to prevent damage.

Collection of *R. cordifolia* Root Powder

R. cordifolia root powder was procured from a reputed century-old certified Ayurvedic medicine shop in Kolkata where most medicines are from plant origin. A qualified botanist of the shop identified the plant. The product was labeled as authentic and sourced from a reputed supplier adhering to Ayurvedic pharmacopoeial standards of India.

Initial experiments for better stain extraction with different chemicals

Initial experiments were done with different chemicals (Table1) for better extraction and accentuation of the colour. In these experiments, we used 0.5mg root powder sample and 3 ml of chemical reagents in each case. After the addition of the chemicals, we observed the colour and their optical density (O.D.) at 530 nm. Details of the results are given in Table 1.

Table 1: Colour changes of Rubia root powder extract with different chemicals.

Reagents	Initial colour	Colour of the supernatant after centrifugation	Colour after 1h	Colour after 2 h
Alcohol (Ethanol or Methanol)	Orange yellow	Orange yellow	Orange yellow	Yellow
Acetic acid (CH_3COOH)	Prawn red	Brownish red	Orange	Orange
20% Sulphuric acid (H_2SO_4)	Orange	Brownish red	Brownish red	Brownish Red
3% Hydrochloric acid (HCl)	Orange yellow	Orange yellow	Light orange	Light Orange
Potassium ferricyanide ($K_3[Fe(CN)_6]$)	Light Grey	Brick red	Brick red	Orange yellow
1N Sodium hydroxide (NaOH)	Blackish red	Blackish red	Blackish red	Blackish Red
10% KOH	Pink red	Pink red	Deep red	Grape red
Benedict solution	Violet red	Deep green	Algae green	Deep green/ Bluish Green
Fouchet's solution	Greenish yellow	Greenish yellow	Greenish brown	Greenish black

In this study, we selected the ethanolic extract. Details of the preparation of this stain are given below.

Novel stain Preparation

5 g of *R. cordifolia* root powder was weighed and mixed with 1 mL of ethanol in a clean Falcon tube. The suspension was mixed in the vortex and then heated in a water bath at 70–80°C for 15–20 minutes to facilitate the extraction of coloured anthraquinones, from the root powder into the solvent. After cooling to room temperature, 2 mL of glacial acetic acid was added to the mixture. The solution was stirred thoroughly to ensure uniform mixing. The prepared suspension was incubated at 37°C in an incubator for 24 hours to enhance the release of dye components and stabilize the staining solution. After that, the incubated mixture was centrifuged at 3000 rpm for 5 minutes, the supernatant was collected in a clean Falcon tube and stored at room temperature for subsequent use in staining procedures. Alcohol followed by acetic acid was used for extraction and mordanting. We observed absorbance in a UV spectrometer at 520 nm wavelength as the extracted colour of ethanol was orange-yellow and that of acetic acid orange. The absorbance with ethanol was 0.4921, while that with acetic acid was 1.0979.

Staining procedure

Besides the conventional H &E method, two other staining methods were used in this experiment.

Hematoxylin *R. cordifolia* (HR) stain method:

A dual staining method combining Harris hematoxylin for nuclear staining with the *R. cordifolia* root powder stain as a substitute for eosin. At first, deparaffinized *Helicobacter pylori* positive histological sections on slides were rehydrated through a graded ethanol series (absolute, 95%, 70%) for 10-15 minutes

each and finally rinsed in distilled water for 2-3 minutes. After that, slides were immersed in Harris hematoxylin solution for 10-15 minutes. Next, excess hematoxylin was washed off under running tap water for 5 minutes. After that, the slides were immersed in the *R. cordifolia* stain for 5 minutes. Excess stain was removed by rinsing the slides in distilled water for 1–2 minutes. Finally, the stained slides were dried and mounted with a resin-based mounting medium DPX, and the stained slides were ready for microscopic examination.

***R. cordifolia* stain method without Harris haematoxylin**:

Only *R. cordifolia* stain was used for the entire staining. At first, deparaffinized *H.pylori* contained histopathological sections on slides that were rehydrated through a graded ethanol series (absolute, 95%, 70%) for 10-15 minutes each and rinsed in distilled water for 2-3 minutes. After that, the *R. cordifolia* stain was applied to the sections of the slides and incubated for 10-15 minutes. Excess stain was removed by rinsing the slides in distilled water for 1–2 minutes. The slides were dried and mounted using a resin-based mounting medium DPX, and finally were ready for microscopic examination.

RESULTS

All *H pylori-positive* cases showed numerous *H. pylori*, particularly in the mucus layer and gastric pits.

The staining with Haematoxylin and Rubia showed excellent results revealing many *H. pylori* that were not visible with routine H&E stain. However, when stained with only Rubia, although some bacteria stained deeply after prolonged staining the staining is not as good as Haematoxylin and Rubia stain (Fig. 1 and Fig. 2). Thus this staining procedure appears ideal for histological staining of *H. pylori*.

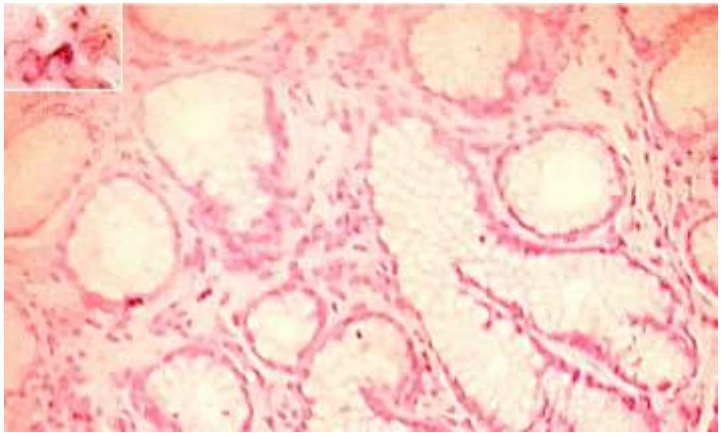


Fig. 1: Haematoxylin and Rubia (H&R) staining shows numerous *Helicobacter pylori* which are stained deeply in comparison to usual H&E staining and delineated clearly for easy diagnosis. Enlarged deeply stained bacteria are shown in the left upper box

Fig. 2: Only Rubia staining without haematoxylin; although *H. pylori* are scattered throughout the microscopic field they are not prominent. Some deeply stained bacteria are shown after prolonged staining (arrow)

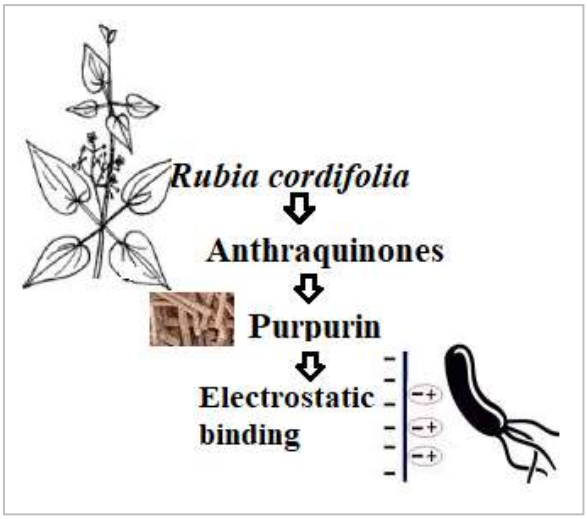
DISCUSSION

Two new staining techniques were employed in this study, and each staining approach produces a different outcome. The staining procedure using hematoxylin for nuclear staining followed by Rubia root powder stain as a substitute for eosin produced distinct and satisfactory results on histopathological slides of *H. pylori*-infected tissue samples. The Rubia stain

effectively highlighted the *H. Pylori* bacteria within the tissue samples when used with haematoxylin. The bacterial structures appeared as numerous small, curved, reddish-orange rods, surrounded by a reddish halo distinctly visible against the nuclei and the lightly stained tissue background. All *H. pylori*-positive cases showed numerous *H. pylori* particularly in the mucus layer and in the gastric pits. The combination of hematoxylin and Rubia stain provided excellent contrast between nuclei, cytoplasm, and bacterial structures. The staining method successfully preserved tissue morphology, allowing clear differentiation of infected areas. The result of this staining technique was most prominent and precise. Compared to synthetic stains, the Rubia stain offered a cost-effective, eco-friendly alternative with comparable diagnostic clarity.

Next, staining of *H. pylori*-positive histopathological sections using Rubia root powder stain without haematoxylin yielded *H. Pylori* bacteria that were visible as small, curved rod-shaped structures within the tissue. The reddish-orange staining of the bacterial cells made them distinguishable from the surrounding tissue components. Minimal background staining was observed compared to Hematoxylin Rubia stain, ensuring a high-contrast appearance of stained bacterial structures only. However, the prepared stain does not offer diagnostic clarity comparable to conventional dyes.

It is important to demonstrate the presence of *H. pylori* in gastric biopsy because all positive cases are treated drastically with antibiotics and proton pump inhibitors [11,12]. Among different methods of detection of *H. pylori*, invasive techniques are more commonly used globally than noninvasive techniques. Important noninvasive techniques are urea breath test, antigen assay in stool, and serum antibody assay. Facilities of some invasive techniques like culture, molecular testing, and immunohistochemistry are costly and available in only a few referral laboratories. Although many studies have indicated that for demonstration of *H. pylori*, H & E staining is as good as all other special stains [4, 13-15], our results indicated that replacing eosin of H& E stain with Rubia root extract showed better and excellent staining of *H. pylori* in the histological sections. The numerous *H. pylori* are not only visible in all the sections but they also become thick with a layering appearance for easy detection even by a non-experienced worker. In this study, we selected cases of chronic active gastritis because in these cases *H. pylori* are commonly found in histological sections. Our findings confirmed that regular H & R (Haematoxylin and Rubia) stained sections are much more effective for the detection of *H. pylori* in histological sections than the routine *H & E* staining and they can be easily identified in x400 magnification. Thus we assert that in all chronic gastritis cases, H & R staining can be performed easily with better results and even in sections with scant organisms this staining method will be an ideal one for all laboratories. The widespread use of proton pump inhibitors leads to a scant number of *H. pylori* in the sections [16]. These bacteria may be converted into coccoid and intracellular forms that may be missed in H&E staining. But with this new staining technique, they become thick and can be visualized easily. After staining with this method, the background is also clear. However, one must be careful with other bacteria and *H. heilmannii*, which may be present in the sections [17]. They usually remain in "clouds" and can be distinguished easily. *H. heilmannii* is elongated, corkscrew-like, and very thick compared to *H. pylori*.



The method of staining by the powdered root extract of *R. cordifolia* is by binding its anthraquinones; with the lipids and proteins, which are present in host tissues and bacterial cell walls (Fig.3). Mainly purpurin of Rubia root extract makes a strong electrostatic binding with Gram negative *H. pylori* cell wall. This approach is helpful in environments with limited resources when accessibility and cost are important factors.

CONCLUSION

The study successfully demonstrated the efficacy of using Rubia root powder stain as a substitute for eosin in histopathological staining of *H. pylori*-infected tissue samples. The dual-staining method, which combined hematoxylin for nuclear staining and Rubia stain for cytoplasmic and bacterial visualization, provides excellent clarity and contrast.

Competing Interests

There is no competing interest of any Author in this manuscript.

Ethical approval

Hospital records without any identity of the patients was used in this study and anonymous sections were used as per Institutional Ethical Committee guidelines.

Author's Contribution

SS and SD designed the study procedure, reviewed and edited the manuscript. ASR and others carried out the experiment, analysed the results and wrote the manuscript.

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