RESEARCH ARTICLE

Modified Toluidine Blue: an Alternative Stain for *Helicobacter pylori* Detection in Routine Diagnostic Use and Post-eradication Confirmation for Gastric Cancer Prevention

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Abstract

**Background:** Modified toluidine blue staining (MTBs) is a simple, inexpensive and time saving method to detect *H. pylori* in gastric biopsy specimens. As a metachromatic stain, it simultaneously highlights intestinal metaplasia, a gastric cancer precancerous lesion. The aim of this study was to assess the reliability of MTBs compared with hematoxylin-eosin (H&E) for *H. pylori* detection using immunoperoxidase staining as the gold standard. This technique would be beneficial for a routine diagnosis and confirmation of *H. pylori* eradication in developing countries where endoscopic-based approaches are dominant. **Materials and Methods:** Esophagogastroduodenoscopy with triple site gastric biopsies was undertaken in 207 dyspeptic patients at Thammasat University Hospital, Thailand between 1997 and 1999. H&E, MTBs and immunoperoxidase staining were applied to each specimen. The presence or absence of *H. pylori* with each stain was interpreted separately and the sensitivity, specificity, positive and negative predictive values of H&E and MTBs were calculated.

**Results:** A total of 282 specimens from 207 patients were evaluated. Using immunoperoxidase staining, organisms were positive in 117 specimens (41%). MTBs proved almost equally sensitive as immunoperoxidase (99%) and significantly more sensitive than H&E (85%). It has comparable specificity (96% vs 96%), PPV (95% vs 94%), and NPV (99% vs 90%) to H&E, using immunoperoxidase staining as gold standard. MTBs compared with immunoperoxidase staining, is cheaper (2 USD vs 12 USD) and faster (20 min vs 16 hrs) compared to immunoperoxidase staining. **Conclusions:** MTBs is effective, economical and easy to use in daily practice for the detection of *H. pylori* in gastric biopsy specimens. In addition to saving time in evaluating *H. pylori* associated gastritis, with a high sensitivity and ability to demonstrate intestinal metaplasia, the technique may have a role in confirmation of *H. pylori* eradication for gastric cancer prevention in a developing country setting.

**Keywords:** *Helicobacter pylori* - histopathology - intestinal metaplasia - modified toluidine blue

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Introduction

*Helicobacter pylori* infection is associated with many gastroduodenal diseases such as peptic ulcer disease and gastric cancer (Uemura et al., 2001). The organism is the major cause of chronic active gastritis and its end stage, gastric atrophy, which is believed to be a precancerous lesion (Pandey et al., 2010). Intestinal metaplasia, usually associated with atrophy, is generally regarded as a condition that is predisposed to malignancy as well (Pandey et al., 2010). Several studies have also shown that *H. pylori* played a major role in the pathogenesis of primary gastric lymphoma (Zullo et al., 2014).

Several techniques are available to detect *H. pylori* infection, including biopsy, culture, PCR, imprint cytology, urea breath test, and serology (Barthel and Everett, 1990; Valentine et al., 1991; Cutler et al., 1995; Matsukura et al., 1995; Thijs et al., 1996; de Boer, 1997). Gastroscopic-based gastric biopsy is one of the most frequently utilized methods. The histologic sections show not only the bacteria but also the morphologic details of the infected mucosa. Although many positive cases can be recognized in a good hematoxylin and eosin (H&E) stain, a special stain for *H. pylori* should be applied before declaring an inflamed biopsy specimen histologically negative for the organisms (Dixon et al., 1996). The choice of stain, for example, modified giemsa, Warthin-Starry, and Genta stain is a matter of local preference (Genta et al., 1994; Dixon et al., 1996; el-Zimaity et al., 1999). Immunohistochemical methods are highly specific and have an important role in specific situations, but cannot be advocated for the routine diagnosis of *H. pylori* gastritis (Genta et al., 1994). Moreover, to detect concomitant
intestinal metaplasia, additional staining may be needed (Wright and Kelly, 2006).

Toluidine blue staining in *H. pylori* study was first reported in the literature by Slater in 1990 (Slater, 1990). Our group has optimized the staining technique and has routinely used the modified toluidine blue stain (MTBs) at Nippon Medical School, Japan and Thammasat University Hospital, Thailand since 1996 (Yali et al., 1997; Dursun et al., 2004; Wright and Kelly, 2006), to our knowledge, there was no study to evaluate the usefulness and the accuracy of this technique. We performed the study to assess the reliability of MTBs compared to H&E stain for *H. pylori* detection in gastric biopsy specimens using the immunohistochemistry against *H. pylori* (immunoperoxidase stain, immunostain) as the gold standard. We proposed the use of this technique for routine diagnosis of *H. pylori* and for confirmation of post-therapy bacterial eradication. The latter may have a role in *H. pylori* screening and eradication strategy for gastric cancer prevention in Asian people (Ford et al., 2014), especially in a tropical developing country like Thailand (Wiwanitkit, 2010).

**Materials and Methods**

Thammasat University hospital is a tertiary care center in Pathumthani, Central Thailand. In general, dyspeptic patients of our hospital were both seen by gastroenterologists (60%) and gastrointestinal surgeons (40%) depended on the referral system. Ten of surgeons have managed dyspeptic patients in our hospital but in this present study, to avoid misclassification bias during endoscopic classification, all dyspeptic patients presented to one surgical endoscopist (P.T.) during a 2-year period (1997-1999) for esophagogastroduodenoscopy were enrolled and consented to the study. The study was approved by Human Research Ethics Committee of Faculty of Medicine, Thammasat University. Data collected included age, sex, past medical history, past history of dyspepsia and medication, smoking and drinking habits, family history and endoscopic findings. At least three fixed points were biopsied at the antrum, corpus and incisura angularis. Specimens were fixed immediately in 10% buffered formalin solution prior to the staining process. Paraffin-embedded sections were cut and stained with hematoxylin and eosin (H&E) and (MTBs). The immunoperoxidase stain against *H. pylori* was applied retrospectively in all unstained sections taken from each set of biopsy specimens. All histologic sections were evaluated by one pathologist (D.S.) who was blinded to the clinical information.

**Modified toluidine blue staining (MTBs)**

For preparation of the MTBs, 50 mg of toluidine blue and 10 ml of 0.1M PBS (pH 6.0) were mixed with 90 ml of distilled water. Staining procedure, using the paraffin-embedded tissue, included deparaffinization, washing in distilled water, then incubated with the stain for 15 min, washing well in water, dehydration, clearing, and mounting.

**Immunohistochemical study**

Sections were cut and deparaffinized with xylene and 100% alcohol and treated with a hydrogenperoxidase-0.3% methanol solution for 30 min. The sections were pretreated with a protease solution (0.1% protease in 0.01 M phosphated-buffered solution (PBS) for 10 min. After cleaning with PBS three times, the sections were incubated with primary antibody against *H. pylori* (polyclonal rabbit anti-Hp, DAKO, California, USA) in a 1:100 solution at 18°C, overnight. The sections were washed three times with PBS and incubated with 1-2 drops of peroxidase-conjugated swine anti-rabbit for 40 min. After washing with PBS three times, the sections were incubated with 3,3-diaminobenzidine tetrahydrochloride (DAB) at room temperature until a dark brown reaction product was obtained, usually after 5-10 min. Counterstaining was performed with hematoxylin.

**Evaluation of the stainings**

The H&E, MTBs and immunoperoxidase stainings of each case were separately evaluated for the presence or absence of *Helicobacter*-like organisms at high power magnification (x400). All specimens were examined blindly without knowledge of the identity of the subjects, their clinical data or other stain results.

**Evaluation of intestinal metaplasia**

In each case, intestinal metaplasia judged to be positive or negative by presenting of intestinal-type epithelium, including goblet cells, absorptive cells and/or Paneth cells on H&E and MTBs-stained slides, respectively.

**Statistical analysis**

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of MTBs and H&E stain for detection of *H. pylori*, using the immunoperoxidase stain as the gold standard, were calculated.

**Results**

**Patient characteristics**

A total of 207 patients were enrolled to the study. Their mean age was 46±17.5 years (range: 16-85). This involved 116 males (56%) and 91 females (44%). Peptic ulcer disease was diagnosed in 81 of the 207 patients (39.1%). There were 43 patients (20.8%) with duodenal ulcer, 30 patients (14.5%) with gastric ulcer and 8 patients (3.9%) with gastroduodenal ulcers. Gastritis was diagnosed in 42 patients (20.3%). In another 61 patients (29.5%), the diagnosis was non-ulcer dyspepsia. The remaining patients were diagnosed as duodenitis (n=6, 2.9%), erosive...
gastrooduodenitis (n=1, 0.5%), gastric erosion (n=6, 2.9%),
hypertensive gastropathy (n=1, 0.5%), gastroesophageal
reflux disease (n=5, 2.4%) and gastric carcinoma (n=4,
1.9%). The total number of endoscopic biopsies in these
207 patients was 282.

The stains
Although visible, *H. pylori* are relatively difficult
to detect with H&E stain. In contrast, they are easily
detected in the MTBs and the immunoperoxidase-stained
sections. For economical evaluation, the immunostaining
process takes 16 hours and costs 12 USD for each staining.
Meanwhile, the whole MTBs staining process will take 20
minutes and the cost of MTBs is 2 USD each.

By MTBs staining, the organisms are dark blue against
a variable blue background. They are typically 0.5 µm
wide, 1-3 µm long, curved spiral-rod in shape confined to
the mucus layer, closely adherent to the mucosal surface,
and/or within the gastric pits (Figure 1). In addition,
the MTBs can simultaneously demonstrate neutrophilic
infiltration in the glands and stroma. The nuclear lobes
of the neutrophils stand out against their clear unstained
cytoplasm.

*H. pylori* were identified on immunoperoxidase-
stained slides in 117 of 282 biopsy specimens (41%). The
organisms stain brown and are located at the same sites
as in MTBs-stained sections. In addition, they are found
in the cytoplasm of neutrophils, focally.

Intestinal metaplasia was identified by H&E and
MTBs in 37 specimens (13%). On MTBs-stained slides,
the metaplastic cells stain magenta contrasting with the
blue background (Figure 2). Although no additional foci
of intestinal metaplasia were seen by MTBs stain, the
metaplastic cells stain magenta contrasting with the
blue background (Figure 2). The specificity and positive predictive value
of MTBs were 99% and 94%; MTBs, 96% and 95%, respectively.

Specimens, which were interpreted differently on
different stains, were carefully reviewed to determine the
nature of the discrepancies. There were 8 positive H&E
stains with negative immunoperoxidase stain. Six of
these cases were positive for MTBs staining. On second
evaluation, two cases with negative MTBs stains were
considered to be false positive H&E stains due to mucus
debris that was erroneously interpreted as *H. pylori*.

Diagnostic test results
The diagnostic accuracy of each stain was shown in

Table 1. Sensitivity, Specificity, Positive and Negative Predictive value of H&E and MTBs for Detection of *H. pylori* Compared with Immunoperoxidase Stain, as the Gold Standard

<table>
<thead>
<tr>
<th>Staining method</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H&amp;E</td>
<td>85</td>
<td>96</td>
<td>94</td>
<td>90</td>
</tr>
<tr>
<td>MTBs</td>
<td>99</td>
<td>96</td>
<td>95</td>
<td>99</td>
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*H&E=hematoxylin and eosin stain; MTBs=modified toluidine blue stain; PPV=positive predictive value; NPV=negative predictive value

Discussion

It should be noted that the importance of detecting *H. pylori* has crossed over from the research laboratory to
clinical practice. The need to eradicate *H. pylori* as part
of the management of peptic ulcer disease and gastric
cancer prevention is increasingly recognized by clinicians.
Meanwhile a new and effective treatment regimen for *H. pylori*
is becoming available, confirmation of eradication
is crucial and is best obtained by the histopathologic
examination of post-treatment biopsy specimens (Genta
et al., 1994).

Toluidine blue staining in *H. pylori* study was firstly
reported in the literature from 1990. Our group from Japan
and Thailand had optimized the staining technique and
used it routinely since 1996. We found that it was a very
easy, fast and cheap for *H. pylori* identification comparing
with other standard techniques. We firstly reported the
use of it in the literature from the year 1998 (Yali et al.,
1998). Nevertheless, one may question the usefulness and
the accuracy of this technique.

In a clinical setting of tropical developing country in
Thailand where availability of Urea Breath Test (UBT)
is still very limited and endoscopic-based diagnosis is
widely used, we present our data of MTBs in comparison
with immunostain as standard. This confirms that our
technique could be used as an alternative technique for
definitive diagnosis of *H. pylori* with a very high economic
benefit. The sensitivity of the MTBs in detecting *H. pylori*
is almost similar to that of the immunostain, which is
currently used as a gold standard in a recent publication
(Yu et al., 2014). Moreover, its negative predictive value

Table 1. MTBs was almost equally sensitive in detecting
*H. pylori* as the immunostain. H&E was less sensitive
(85%) than MTB (99%) using immunostain as gold
standard. MTBs also had higher negative predictive value
(99%) in determining *H. pylori* infection than H&E (90%).

Figure 1. *H. pylori* are typically Curved Spiral-Rod in
Shape, Confined to the Mucus Layer, Closely Adherent
to the Mucosal Surface and within the Gastric Pits.
(MTBs, original magnification x400)

Figure 2. The Metaplastic Cells Stain Magenta, in
Contrast to the Blue Background Indicated Intestinal
Metaplasia. (MTBs, original magnification x400)
of 99% indicates that it is the reliable method to exclude *H. pylori* infection, if negative. The limitation of this study is the sensitivity and specificity of MTBs may vary among different observers depending on the experience of pathologists in identifying the organisms.

MTBs enhances the visualization of bacteria, particularly in a specimen with low density of *H. pylori*, including the post-eradication cases, in small-sized biopsies containing few glands, and in case of abundant mucus debris on the surface or pits. The stain also highlights the neutrophilic infiltration which is highly associated with *H. pylori* infection. Furthermore, the cost of the reagents is much lower than that of Warthin-Starry and immunoperoxidase stains. The staining process is also simpler and the time needed for testing is reduced (within 20 minutes). We propose the use of MTBs technique as a cost-effectiveness tool for *H. pylori* diagnosis and underlying the use in a follow-up period after bacterial eradication in gastric cancer prevention strategy for Asian people (Ford et al., 2014), especially for a clinical setting in developing country (Wiwanitkit, 2010).

There are two possible explanations for the discrepancies between MTBs and immunoperoxidase stain: the patchy distribution of *H. pylori* in gastric samples and/or focal loss of the organisms during the staining process. Therefore, to avoid such false negative results, adequate specimens are necessary. For adequacy, specimens from antrum together with corpus and incisura angularis are recommended. Corpus biopsies are particularly valuable for yielding positive results after treatment, especially where proton pump inhibitors have been used. Under these circumstances, organisms may become rare or disappear from the antrum but remain in the oxyntic mucosa. However, maximal degrees of atrophy and intestinal metaplasia are consistently found in the region of the incisura angularis, which is also the site most likely to reveal premalignant dysplasia (Dixon et al., 1996).

Many laboratories routinely employ an Alcian blue (pH 2.5)/periodic acid Schiff stain on gastric specimens to demonstrate intestinal metaplasia (Dixon et al., 1996). Acid mucin in metaplastic cells stains blue or purple of the Alcian blue, in contrast to the Schiff-positive neutral mucin presented in the surface and foveolar epithelium and the mucus glands of the non-metaplastic gastric mucosa. In light of the knowledge that acid mucin can be demonstrated by the metachromatic stains (Cook, 1996), MTBs with its metachromatic property, can be applied for effective demonstration of intestinal metaplasia. Using the stain allows simultaneous evaluation of *H. pylori* infection status, gastritis activity and intestinal metaplasia on a single slide. Although MTBs does not enhance the sensitivity for detection of intestinal metaplasia, it is easily recognizable and provides a more precise assessment of the extension.

In conclusion, MTBs is effective, economical and easy to use in daily practice for detection of *H. pylori* on gastric biopsy specimens in addition to routine histologic sections. With its high sensitivity to the organisms and the ability to demonstrate intestinal metaplasia and to highlight the neutrophilic infiltration, it lessens the time necessary to evaluate *H. pylori*-associated gastritis. MTBs may be beneficial for confirmation of bacterial eradication for gastric cancer prevention in developing country.

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Part of this work was presented at XXV Congress of the International Academy of Pathology 2004 in Brisbane, Queensland, Australia and the abstract of this work is published in Pathology International, 2004, Vol. 54 (Supple. 2), page A82-A83. The reason for the delay in publishing the full text is that difficulties were encountered in compiling the collaborative work of the various investigators, due to the retirement and/or relocation of some of them.

**References**


el-Zimaity HM, Wu J, Graham DY (1999). Modified Genta triple stain: the patchy distribution of results, adequate specimens are necessary. For adequacy, specimens from antrum together with corpus and incisura angularis are recommended. Corpus biopsies are particularly valuable for yielding positive results after treatment, especially where proton pump inhibitors have been used. Under these circumstances, organisms may become rare or disappear from the antrum but remain in the oxyntic mucosa. However, maximal degrees of atrophy and intestinal metaplasia are consistently found in the region of the incisura angularis, which is also the site most likely to reveal premalignant dysplasia (Dixon et al., 1996).

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Modified Toluidine Blue as an Alternative Stain for \textit{H. pylori} Detection


