Acridine orange stain in the histological identification of Helicobacter pylori

The recent paper by Rotimi and colleagues does not mention the acridine orange stain when comparing staining methods for the identification of Helicobacter pylori. The acridine orange stain uses ultraviolet fluorescence in the identification of bacteria. The morphology of H pylori can easily be differentiated from other bacteria. I have used this quick, cheap, and reliable stain in routine histopathology reporting for over 16 years and it has proved to be extremely useful in the identification of H pylori.

Immunohistochemistry is now recognized as the "gold standard" because it is a highly sensitive and specific staining method. After the publication of the above mentioned article, 20 consecutive gastric biopsies that were positive for H pylori using the acridine orange stain were also stained using the polyclonal anti-H pylori antibody (Dako, Ely, Cambridge, UK) at a dilution of 1/100. Twenty negative control cases were similarly studied. All 20 cases that were positive with the acridine orange stain were also positive by immunohistochemistry and all negative cases were also negative by immunohistochemistry.

This small study clearly shows that ultraviolet fluorescence of H pylori using the acridine orange stain is highly sensitive and compares equally with the gold standard of immunohistochemistry. The acridine orange stain may not be specific, but the morphology of H pylori is clearly visible down to the single organism (fig 1).

The only disadvantage of the acridine orange stain is that the microscope needs a fluorescent attachment, which in my laboratory means turning the lever on a Leitz Diaplan microscope to the required position, without the need for a dark room.

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The authors reply

In pointing out that we omitted to include acridine orange, in our comparison of histological stains for Helicobacter pylori, Dr Haqqani seems to have misunderstood the aim of our study. We sought to compare two recently described staining techniques for which there had been claims of superiority over routine methods with our own previously validated routine stain, the modified Giemsa, and with immunohistochemistry using an anti-H pylori serum. We acknowledged histological "gold standard". There was no attempt to be comprehensive and test every possible stain for H pylori. Thus, we also omitted from our study the variants of the Gram stain used by some laboratories—the Brown-Hopps, Brown-Brenn, and the half Gram—the simple and inexpensive cresyl fast violet and carbol fuchsin stains, and the more elaborate Gimenez stain. The silver impregnation Warthin Starry stain is also used, but is somewhat inconsistent in our hands. Similarly, we have had problems reproducing the silver based Genta "triple" stain. We have no experience of the more recently described techniques of acridine orange–alcian blue–haematoxylin and eosin, and the alcian blue–toluidine blue methods, which could have been added to the panel. It is evident that stains for H pylori have become a cottage industry in which laboratories strive to produce a novel tinctorial mélange, with (in many cases) little thought for sensitivity, specificity, ease of use, reproducibility, and cost.

As is apparent, Dr Haqqani has had a long experience of acridine orange as his preferred routine method. Indeed, by introducing this approach before 1985 he anticipated the need for routine histological assessment of H pylori status before many laboratories (including our own), and antedated the first published report of the use of acridine orange for this purpose in 1986. We are happy that he finds acridine orange a good method in his hands and we concurred in our experience which led us to conclude that it had no particular advantage over the modified Giemsa. Large numbers of organisms within the mucous layer (as shown in fig 1) are readily seen, but we found that scanty numbers of organisms close to the fluorescent gastric epithelium were difficult to discern. We also felt it inappropriate to promote a fluorescent staining method as a routine approach when it requires an ultraviolet light source to be fitted to the microscope, notwithstanding the ease with which Dr Haqqani brings this into action.

However, the claim made by Dr Haqqani that acridine orange is "highly sensitive and compares equally with the gold standard" has to be challenged. To take 20 cases declared positive using the acridine orange stain and then declaring them negative with the "gold standard" immunostain offers no validation whatsoever. The most insensitive of stains would pass this test. Likewise, no conclusions can be drawn from re-testing 20 acidine orange negative "control" biopsies. An accurate estimate of sensitivity and specificity can only be obtained by testing large numbers of unselected cases verified as positive or negative by non-histological methods. We would like to re-emphasize that in an earlier study based on 520 patients who had their H pylori status validated by urea breath test, biopsy urease test, and culture, the modified Giemsa had a sensitivity of 98.8% and a specificity of 99.2%. Although this amply justifies our own confidence in the modified Giemsa stain, these results may not be reproduced by different pathologists serving different populations. As stated previously, the choice of stain is a matter of personal judgement and laboratory practice. The most valuable requirement is for diligent, enthusiastic histopathologists who can recognise helicobacters by whichever stain they choose.

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Formalin or not formalin; that is the question

We have all faced the dilemma. The laboratory receives a universal container in which a tissue sample is immersed in a clear liquid. The tissue is still pink and blood stained. So has the sample been placed in saline in error or is it in formalin and simply not yet fixed? In time honoured fashion, the laboratory technican or pathologist removes the lid of the container and gingerly inhales. Regrettably, by the time the characteristic odour of formalin is recognised, its noxious and irritating properties have already inflicted their damage upon the tearful eyed investigator. It need no longer be this way!

An easily and rapidly applied technique can establish the presence or absence of formalin without placing the investigating staff in harm's way.

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Figure 1 Helicobacter pylori stained with acridine orange. Original magnification, ×250.


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An easily and rapidly applied technique can establish the presence or absence of formalin without placing the investigating staff in harm's way.
Place a few drops of reticulin solution in a beaker and add small drops of your test solu-
tion. If the test solution is formalin, the reticulin solution will turn black. A similar
effect can be produced by adding the test solution to Schiff’s solution. In this case, add-
ing drops of formalin will turn the combina-
tion a deep magenta colour. The addition of a test solution of saline (the most frequently
encountered alternative) will produce no col-
our change to Schiff’s solution and will turn reticulin solution white. Because all laborato-
ries will have both reagents already prepared on their shelves, the test may be done in a
matter of seconds.

Good old fashioned chemistry to the rescue!

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Cytokeratin expression by CD34
positive blasts in a case of refractory
anaemia with excess of blasts in
transformation (RAEB-t)

Immunohistochemistry has become a very
important, and in some cases indispensable,
tool in diagnostic pathology, enabling the
precise identification of tumours, the detec-
tion of micrometastases in a given sample,
and the evaluation of various prognosis
factors. However, in some cases, the use of
multiple but distinct immunostains can lead
to some unforeseen results—for example, the
expression of an apparently aberrant marker
by a neoplasm can sometimes be seen. In this
context, we report our experience with a case of refractory anaemia with excess of blasts in
transformation (RAEB-t) in which the blasts
were unexpectedly found to express cytoker-
tin (CK).

An 86 year old woman with a past medical
history of breast carcinoma treated by
mastectomy and adjuvant radiotherapy was admitted to our institution because of wors-
eining anaemia. The following haematologi-
cal indices were noticed: haemoglobin, 8.6 g/
litre; erythrocytes, 2.5 × 10¹³/litre; white
blood cells, 3 × 10⁹/litre; and platelets,
465 × 10⁹/litre. Blasts were also found in
peripheral blood (11%). Both bone marrow
aspirate and biopsy demonstrated features
of refractory anaemia with excess of blasts in
transformation (RAEB-t) as identified as an RAEB-t
according to the criteria of the French–
American–British (FAB) group.

Cytochemical study of the blasts revealed the
presence of α-naphthyl acetate esterase but
not of myeloperoxidase (MPO) or naphthyl
ASD chloroacetate esterase. On flow cyto-
meter, the blasts were found to be CD34
positive with expression to some extent of
CD13, CD33, CD45, CD45RA, CD117
(c-kit gene product), and CD35. No expres-
sion of CD10, CD19, CD45RO or CD90
could be demonstrated. Immunohisto-
chemical studies performed on paraffin wax
embedded sections demonstrated CD34 and
CD45 positivity in the blasts (fig 1A). How-
ever, these cells were MPO, CD3, and CD20
negative. CD68 expression was variable.
To exclude with certainty the possibility of an
unnecessary bone marrow infiltration by the
underlying breast carcinoma, complement-
ant anti-CK stains using KL-1 and CAM
5.2 antibodies were performed. Surprisingly,
the blasts showed a strong perinuclear or
punctuate (dot-like) staining pattern (fig
2A). However, these cells did not react with
the anti-CK19 antibody, further demonstrat-
ing the absence of bone marrow infiltration by
the breast carcinoma.

CK expression by myeloid blasts is a very
uncommon finding—only three publications
(two case reports and one in vitro study)
dealing with this matter have been published
so far.4 In this setting, the comparison
between our findings and those described in
these reports allows us to make some
interesting comments. First, similar to CK
expression reported in various lymphomatous
and plasma cell disorders,1 the immunohisto-
chemical pattern of CK expression in myeloid
blasts is also dot-like or perinuclear.
Another similarity between these studies and
our case is the use of antibodies that recognises a wide spectrum of CKs (preker-
tin, KL-1, AE1/AE3 cocktail, or CAM
5.2).4,5 Indeed, the KL-1 antibody reacts
with the following CK polypeptides: CK1,
CK2, CK5, CK6, CK7, CK8, CK11, CK14,
CK16, CK17, and CK18. The antibody
cocktail AE1/AE3 recognises numerous
acidic and basic keratins, namely: CK10,
CK14, CK15, CK16, CK19 and CK1, CK2,
CK3, CK4, CK5, CK6, CK7, CK8, respec-
tively. Finally, CK8 and CK18 are labelled
by the CAM 5.2 antibody. In this regard, it is
worth noting that these blasts are stained by
AE1 but not by AE3 when these antibodies
are used separately.6 Another point of
interest is the similar differentiation that
characterises CK expressing blasts. Indeed,
the two reported cases of CK positive acute
myeloid leukaemia (AML) reported be-
longing to the category of AML FAB M4.7
Although precise subtyping of AML arising
from MDS may be difficult, the morphologi-
ical, cytochemical, and phenotypic features
seen in our patient are also consistent with a
myelomonocytic differentiation. Like Turner
and Milliken,8 we found no CK19 expression
by the blasts. Therefore, this observation
shows that CK19 is a more specific marker of
carcinomas showing glandular differentia-
tion than are various pan-CK immunos-

tains. What the precise explanation for
this unusual phenomenon may be, in
addition to the two cases published previ-
ously, our observation illustrates that CK
expression can be seen, albeit rarely, in
AML.

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Audit of colposcopy biopsy sectioning

In April 1999, the guidance document
“Histopathology reporting in cervical screen-
ing” was issued.1 On page 45 of that
document is the statement “As the appear-
ance of the tissues, even in small biopsies,
often shows considerable variation, several
levels are required to ensure that small foci of
disease are identified”. No more specific
guidance was given in the document. The
wholly of what constitutes “several levels” was
discussed at the Symposium of Gynaecologi-
cal Pathology held by the British Division of the
International Academy of Pathology in
Sheffield on 10 September 1999. It was clear
from the discussion that there were many
varying practices being used. The practice in
the laboratory at the City Hospital Nottingham was to examine two sections at each of three levels of the material, all mounted on to one glass slide. It was decided that this practice should be audited against examining two sections at each of six levels to see whether extra information was gained by this or whether important diagnostic features were being missed by using the existing practice. The subsequent consecutive 100 colposcopic biopsies were processed according to the standard operating procedure in the laboratory and then two sections 2 µm thick were cut at each of six levels through the material. The levelling was rigorously controlled at 50 µm between each level. The levels were mounted as sections one to three on one slide and sections four to six on a second slide.

The samples were examined microscopically (all by JJ). The slide with levels one to three was examined and the diagnostic features recorded. Only then was the slide with the levels four to six examined. Any variance from the features seen in the slide with the levels four to six examined. The conclusion reached has been to continue our original practice, thereby preventing the use of extra sectioning time and of twice the number of slides for each case. Critical evaluation of one versus two sections from each level was not conducted but one section would save very little cutting time and six sections sit easily on one slide.

During the course of our study, a letter appeared in the Journal of Clinical Pathology stating that sections at levels through the tissue should not be mounted on the same slide because the histological material may not be covered by the coverslip, or might be obscured by mountant. This was not the case on any of our 200 slides. With careful placing of the material on the slide by the section cutter (fig 1) and an automated coverslipping machine the artefacts mentioned in that letter are not encountered.

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Figure 1 Three slides showing two sections at each of three levels mounted on one slide. The first level is closest to the label. The ink marks have been put on to the coverslip by the pathologist to encircle the material for examination at each level and to guide the eye from level to level when examining the material under the microscope (especially useful when the levels are offset on the slide).

Cytokeratin expression by CD34 positive blasts in a case of refractory anaemia with excess of blasts in transformation (RAEB-t)

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