

The Warthin-Starry stain in the diagnosis of small intestinal microsporidiosis in HIV-infected patients

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Abstract. A protocol for the handling of small intestinal biopsies from HIV-infected patients is presented. This protocol includes the Warthin-Starry stain for the detection of microsporidia. This stain has proved a reliable and sensitive diagnostic technique for microsporidial infections as it stains both *Enterocytozoon bieneusi* and *Septata intestinalis* in duodenal enterocytes. Because the stain demonstrates *Septata intestinalis* in lamina propria macrophages as well as enterocytes, it allows for the practical differentiation of these two microsporidial infections. The Warthin-Starry stain has also demonstrated *Septata intestinalis* in nasal and colonic biopsies in some of these patients.

Since the completion of an earlier study, a further 40 cases of *Enterocytozoon bieneusi* and three cases of *Septata intestinalis* have been diagnosed in just over 240 consecutive duodenal biopsies from HIV positive patients presenting with diarrhoea and other gastrointestinal complaints. Other opportunistic infections include cytomegalovirus in four cases, mycobacteria in eight cases, cryptosporidia in nine cases, giardia in four cases and *Isospora belli* in one case. Since the ratio of these opportunistic infections has remained much the same as in the previous study of 180 consecutive duodenal biopsies, we suggest that these rates may reflect the actual prevalence of microsporidial infections in AIDS patients in Sydney, Australia.

The original diagnosis of *Enterocytozoon bieneusi* in 1985 (Desportes et al. 1985, Dobbins and Weinstein 1985) relied on electron microscopy and introduced the microsporidian as the fourth genus implicated in human pathology, the other three genera being *Pleistophora*, *Nosema* and *Encephalitozoon* spp. Since that time a number of studies have presented the pathology and attempted to delineate the incidence and prevalence of this infection in HIV-infected patients. Although these studies have used paraffin embedded, hematoxylin and eosin stained small intestinal biopsies as a guide, the routine diagnosis has relied on resin embedded sections and electron microscopy (Rijpstra et al. 1988, Lucas et al. 1989, Canning and Hollister 1990, Orenstein et al. 1990, Curry et al. 1991, Peacock et al. 1991, Eeftinck Schattenkerk et al. 1991).

Recently a second microsporidian, *Septata intestinalis* has been described involving duodenal and small intestinal biopsies in HIV-infected patients (Orenstein et al. 1992, Cali et al. 1993). Although this microsporidian infection appears to be more easily diagnosed in hematoxylin and eosin stained paraffin embedded sections of the small bowel, final diagnosis again has rested on electron microscopy or at least resin embedded sections.

As we have previously reported (Field et al. 1993a,b) the Warthin-Starry stain applied to sections

of paraffin embedded duodenal biopsies is a reliable diagnostic technique for both *E. bieneusi* and *S. intestinalis*, obviating the requirement for resin embedded sections or routine diagnostic electron microscopy.

We present our protocol for handling duodenal biopsies from HIV-infected patients, our method for the Warthin-Starry stain, and present an update on the relative incidence of the various opportunistic infections found in duodenal biopsies using this protocol.

MATERIALS AND METHODS

Since the completion of our earlier study (Field et al. 1993b) just over a further 240 consecutive duodenal biopsies from HIV-infected patients have been handled in our laboratory using the protocol we have described. Multiple duodenal biopsies obtained from the second part of the duodenum, are fixed in phosphate buffered formalin, embedded in paraffin, and examined at three levels using the hematoxylin and eosin stain. Extra slides from level 2 are cut and stained with the Warthin-Starry stain for microsporidia, the auramine stain for mycobacteria and a CMV immunoperoxidase study (MAB 810, Chemicon International, Temecula, California). In our earlier study (Field et al. 1993b), 100% concordance was shown between light microscopy using the H&E and Warthin-Starry stains, and electron microscopy. Currently, electron microscopy is not used for the routine diagnosis of *E. bieneusi* or *S. intestinalis*, although all of the latter cases diagnosed using light microscopy, have undergone electron microscopy as part of our ongoing research.

RESULTS

In the 15 months since the completion of our initial study a further 40 cases of *E. bienewsi* have been diagnosed from just over 240 consecutive duodenal biopsies. *S. intestinalis* was found in a further three cases, cytomegalovirus in four cases, mycobacteria in eight, cryptosporidia in nine, giardia in four and *Isospora belli* in one. Including the original reported series, a total of 73 *E. bienewsi* cases, 7 *S. intestinalis*, 15 cytomegalovirus, 16 mycobacterial, 17 cryptosporidial, 9 giardia and one *I. belli* infection have been diagnosed out of a total of just over 420 duodenal biopsies.

Light microscopy

The low power appearance of the hematoxylin and eosin (H&E) stained sections shows short, broad villi which are irregular in shape, along with mild crypt hyperplasia, disorganisation of the superficial enterocytes and mildly increased intraepithelial lymphocytes especially over the tips of the villi, and a mild to moderate increase in chronic inflammatory cells and occasional neutrophils in the lamina propria. Macrophages sometimes containing considerable debris, can be prominent in the lamina propria of the tips of the villi especially in the cases of *S. intestinalis* (Fig. 1). We have not seen necrosis or ulceration of the duodenum. The low power appearances are nonspecific, but should alert the pathologist to the fact that significant pathology is present, almost always associated with an opportunistic infection.

High power magnification (x400, x600 dry, x1000 oil immersion) confirms the disorganisation of the superficial enterocytes over the tips of the villi, with increased intraepithelial lymphocytes (Fig. 1). In the case of *E. bienewsi* vacuoles representing plasmodia often are seen indenting the luminal aspect of the enterocyte nuclei, and these vacuoles may contain granular or striated basophilic material. At times these vacuoles can be scarce and may mimic degenerate goblet cells. In both *E. bienewsi* and *S. intestinalis*, oval granular aggregates of developing organisms can be seen in the superficial cytoplasm of the enterocytes. This basophilic granular material can indent the nuclei, but at times can be difficult to differentiate from lysosomal debris. At highest magnification (oil immersion, x1000), spores can sometimes be recognized, especially with *S. intestinalis* whose pink to red spores are larger and slightly birefringent in the H&E stain.

Degenerate enterocytes containing recognizable spores can be seen desquamating from the surface of the epithelium. Only very occasionally in *S. intestinalis* infections can examination of the macrophages in the superficial lamina propria suggest the presence of parasitophorous vacuoles and spores.

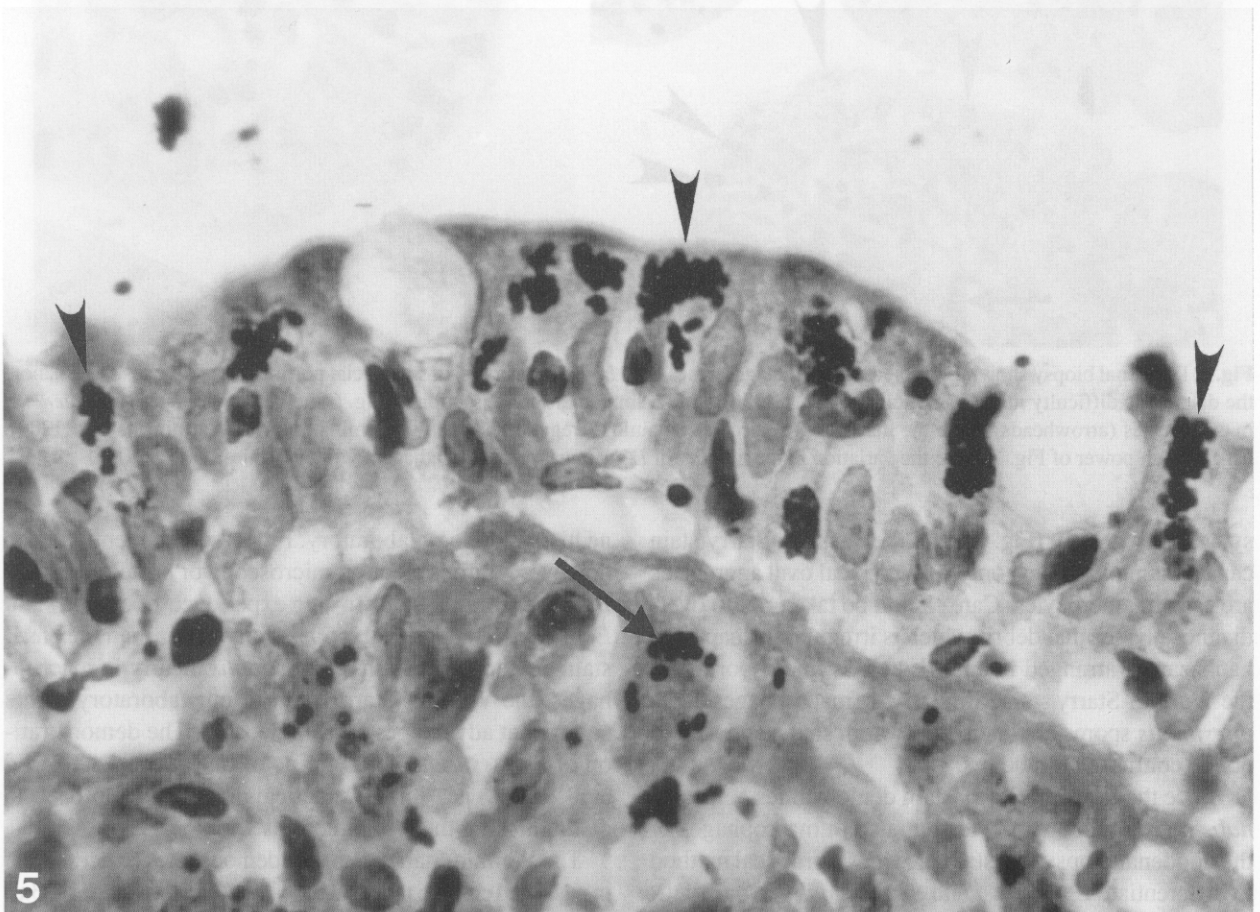
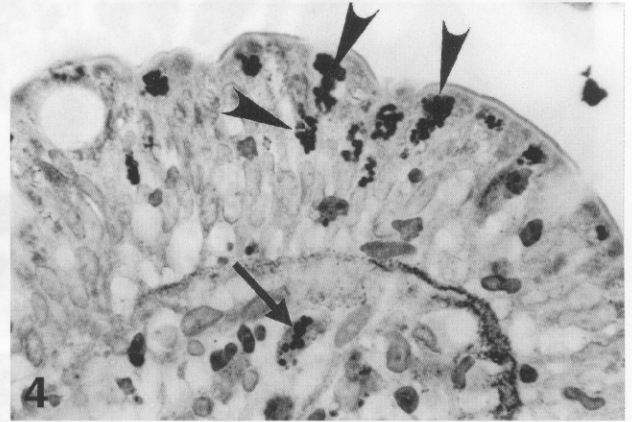
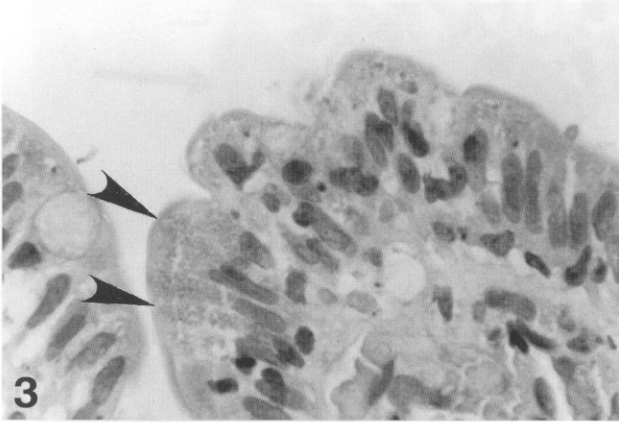
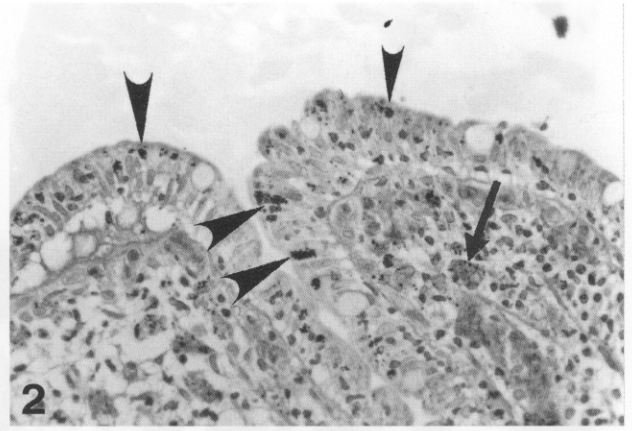
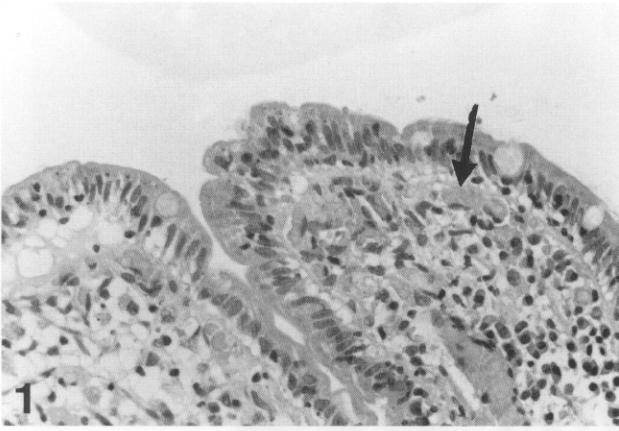
The Warthin-Starry stain of paraffin embedded duodenal sections in cases of *E. bienewsi* and *S. intestinalis* clearly shows oval aggregates of spores and spore precursors in the superficial aspect of the enterocyte cytoplasm indenting the nucleus (Figs. 2, 4, 5, 7, 8). Occasionally in *S. intestinalis* a parasitophorous vacuole containing the spores can be recognized under oil magnification (x1000). The earlier stages of the plasmodia of *E. bienewsi* can be recognized in the Warthin-Starry stain as light to dark brown to black, stippled or "mulberry"-shaped aggregates in the enterocytes (Figs. 7, 8). As the organism matures, distinct spores can be recognized throughout the enterocyte cytoplasm. Spores can be seen as well on the surface of the epithelium. With *S. intestinalis* the spores are larger, with a distinct oval shape and a cleared centre, forming large oval aggregates or dispersed within the enterocytes (Figs. 4, 5). In addition, the Warthin-Starry stain clearly shows oval *S. intestinalis* spores in the cytoplasm of macrophages in the superficial lamina propria, either dispersed or in aggregates, presumably parasitophorous vacuoles (Figs. 4, 5).

Gram's stain stains the spores and developing precursors of *S. intestinalis* in enterocytes reasonably well, but in our experience does not stain any of the stages for *E. bienewsi* better than the H&E stain. Further, Gram's stain does not distinguish spores in macrophages in the lamina propria from Gram's staining debris. Similarly the mild birefringence of the spores is not of great use, since debris within the macrophages also can be slightly birefringent.

DISCUSSION

Since the completion of our initial study (Field et al. 1993a,b), we have continued to use the Warthin-Starry stain in our routine diagnostic protocol for duodenal biopsies from HIV-infected patients. We have found the stain to be a reliable and sensitive method for the identification of microsporidia. Multiple levels of multiple biopsies (routinely three duodenal biopsies are taken) can be quickly assessed in cases where microsporidia are infrequent. The number of microsporidia involving each villus or individual biop-

Fig. 1. Duodenal biopsy from a case of *Septata intestinalis* showing disordered enterocytes with increased intraepithelial lymphocytes and increased chronic inflammatory cells and macrophages (arrow) in the lamina propria (H&E, x800). **Fig. 2.** Immediate serial section of same duodenal biopsy as Fig. 1, stained with the Warthin-Starry stain, showing oval aggregates (arrowheads) of *S. intestinalis* in enterocytes and spores (arrow) in macrophages (Warthin-Starry, x800). **Fig. 3.** High power of Fig. 1 shows granular aggregates of *S. intestinalis* (arrowheads) in enterocytes. Note the diagnostic difficulty in the H&E stain. (H&E, x2000). **Fig. 4.** High power of duodenal biopsy showing oval aggregates of *S. intestinalis* in enterocytes (arrowheads) and spores (arrow) in a macrophage (Warthin-Starry, x2000). **Fig. 5.** High power of duodenal biopsy showing aggregates of *S. intestinalis* in enterocytes (arrowheads) and a macrophage (arrow) as well as dispersed oval spores (Warthin-Starry, x4000).



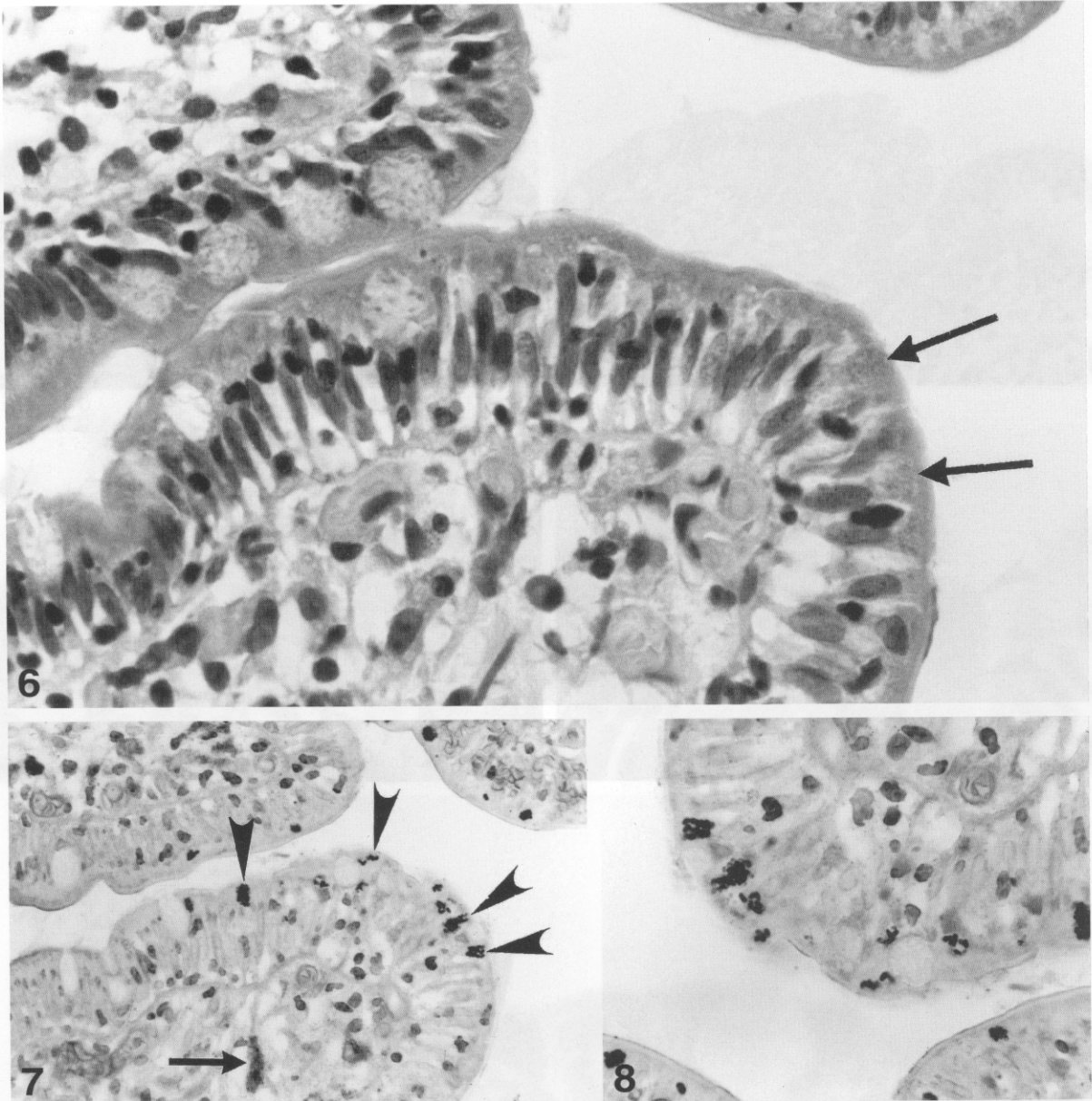


Fig. 6. Duodenal biopsy showing stippled basophilic aggregates of *E. bienewisi* (arrows) in superficial portion of enterocytes. Again, note the diagnostic difficulty in the H&E stain. (H&E, x4000). **Fig. 7.** Immediate serial section of Fig. 6 showing aggregates of *E. bienewisi* in enterocytes (arrowheads). Note the macrophage (arrow) containing irregular debris in the lamina propria (Warthin-Starry, x1200). **Fig. 8.** High power of Fig. 7. Note the variation in the number of *E. bienewisi* present in adjacent villi (Warthin-Starry, x2000).

sy can vary greatly (Fig. 8), and the Warthin-Starry stain can be diagnostic when only a few typical oval aggregates are seen in enterocytes. Care should be taken not to over-diagnose lysosomal debris which is irregular in shape and haphazardly arranged in enterocytes, as microsporidia in the Warthin-Starry stain. Similarly, in macrophages the *S. intestinalis* spores are distinctly oval in shape and should not be confused with debris.

Since the Warthin-Starry stain demonstrates *S. intestinalis* not only in enterocytes but also in macrophages of the duodenal biopsy, the stain provides a practical method to differentiate *E. bienewisi* and *S. intestinalis* in our rou-

ne histopathology laboratory. Final species identification however requires electron microscopy or protein profiles. Because Gram's stain in our experience does not stain *E. bienewisi* in duodenal biopsies any better than the H&E stain and does not stain *S. intestinalis* clearly in macrophages, the Warthin-Starry stain in our laboratory offers significant advantages as a single stain. The demonstration of *S. intestinalis* in the urine of our patients (Field et al. 1993a) is a further practical diagnostic approach to distinguishing the two infections.

The Warthin-Starry stain also demonstrates *S. intestinalis* in nasal and colonic mucosal biopsies (Field et al. 1993a).

The relative frequency of the opportunistic infections identified in the duodenal biopsies of the HIV-infected patients has remained the same when comparing the initial series and our subsequent cases. In all cases of microsporidiosis the patients presented with diarrhoea and wasting, except for the one case in the original series (Field et al. 1993b) who presented with abdominal pain and has remained free of diarrhoea to this time. Since the source of patients has not varied to any great extent,

and the clinical practice and diagnostic protocols of the clinicians have not changed, we suggest that our results may well indicate the prevalence of microsporidia in the HIV-positive community in Sydney, Australia.

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APPENDIX

Warthin-Starry stain: from AFIP Manual of Histologic Staining Methods (Luna 1968) with modifications by the Department of Anatomical Pathology, St Vincent's Hospital, Sydney.

Method:

1. Take sections from paraffin blocks to distilled water including a known positive control for helicobacter or spirochaetes or microsporidia.
2. Rinse slides back and front thoroughly with distilled water. Place sections into freshly prepared 1% silver nitrate for 30 min at 37°C.
3. Wash in distilled water 3 changes.
4. Place sections into the developing solution (see below) in a 60°C waterbath until the sections turn a light brown (approx. 2 to 3 min - see below).

5. Check microscopically for the right result.
6. Rinse well in distilled water, then hot running tap water, then distilled water.
7. Fix sections in 5% sodium thiosulphate for 3 min.
8. Wash well in water for 2 min. Dehydrate, clear in xylol and mount in Eukitt immediately to prevent fading.

Results: Microsporidia, spirochaetes and helicobacter are dark brown to black, with the background a light golden brown.

Solutions:

1. 1% silver nitrate (AgNO_3)
Dilute 1:10 from stock 10% silver nitrate with acidified water pH 4.0.

2. Developing solution:

Quantity for coplin jars 50 ml

Water pH 4.0	15 ml	Mix and pre-heat
and 5% gelatine	18 ml	in 60°C waterbath.
10% silver nitrate	1.5 ml	Add just before
and 1.5% quinol	1.0 ml	developing.

Note: Quinol (also known as 1.5% hydroxyquinone) should be freshly made or not more than one week old.

NB: There are three major problems that occur with the stain:

1. dirty background,
2. the golden yellow colour of the background and dark brown to black colour of the organisms are not achieved,
3. the fading of stains.

Methods to minimise these problems:

1. To prevent the dirty background:

- (i) Do not use any adhesive e.g. polylysine, gelatine, albumin, since these attract the silver stain leaving a deposit on the slide.
- (ii) Ensure that all the glassware used, especially glass slides, is clean and free from any contaminants at all stages.

(iii) Distilled water is essential for:

- (a) making solutions,
 - (b) rinsing glassware.
- (iv) Use high quality reagents (e.g. Analytical Reagent grade).
 - (v) Determination of the end point of colour development is vital since the change in colour from yellow to brown background is rapid (between 2 1/2 and 3 1/2 minutes) and easily missed unless the slides are carefully watched.

2. To achieve the brightness of golden brown colour:

- (i) Acidulated water (pH 4.0) is recommended (pH with citric acid, do not use acetic acetate buffer).
- (ii) Working solutions must be fresh. The quinol is most critical - a development of yellow tea colour in the background indicates oxidation and the quinol should be discarded.

3. To prevent fading:

- (i) Stained sections should be treated with 5% sodium thio-sulphate.
- (ii) Xylol should be used as the clearing agent.
- (iii) Mounting medium is also important (Eukitt is recommended).
- (iv) Sections should be mounted immediately after staining.

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