Silver Impregnation Stain for
Leptospira and Flagella

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Leptospires are difficult to stain with the usual bacterial stains; many different stains have been proposed, most of which are designed for staining of tissue sections. Silver stains that are available for smear preparations yield variable, unreliable results. Staining of bacterial flagella is often avoided because of difficulties involved. There exists a need for a simple technique which does not greatly alter the morphology of the leptospiral cell, or which will reliably stain flagella.

The silver impregnation technique described here is a modification of a technique described by D. S. Kim of the Veterinary Research Institute, Pusan, Korea (unpublished data). The modified technique is rapid, employs only two reagents which are easily applied, and requires no special fixation or heating. Alteration of the morphology of leptospires and flagella is minimal. The spiral structure of the spirochetes can often be seen in a relatively normal state.

Reagent A is composed of 100 ml of distilled water containing 5 g of tannic acid, 1.5 g of ferric chloride, 2.0 ml of 15% formalin, and 1.0 ml of 1% sodium hydroxide.

Reagent B, ammoniated silver nitrate solution, is prepared by use of 100 ml of 2% silver nitrate. About 10 ml of this volume is removed and saved; to the remaining 90 ml, ammonium hydroxide is added dropwise until the heavy precipitate that is formed is dissolved. From the 10 ml previously removed, 2% silver nitrate is added dropwise until a slight clouding appears and persists. At this point, the pH is adjusted to 10.0 with the ammonium hydroxide and silver nitrate. Reagent B is relatively unstable, and must be used within 4 hr of preparation. Readjustment of the pH to 10.0 after it changes has not produced satisfactory results.

Smears are prepared in a routine fashion except that extreme caution must be employed in handling bacterial suspensions for flagella stain. The slides are alcohol-cleaned and a loopful of distilled water is placed on each. A loopful of culture or suspension is placed just touching the distilled water, so that the two diffuse together to achieve a gradation of dilution of the medium. Slides are then allowed to air-dry. Fixation by heat is unnecessary.

For the preparation of bacteria to produce and maintain maximal numbers of flagella, incubation at 20 C, harvesting organisms during the logarithmic or early stationary phase, preparation of a faintly cloudy suspension in distilled water, and extreme care in handling, all as described by Leifson (Atlas of Bacterial Flagellation, Academic Press, Inc., New York, 1960) are essential.

Blood films from animals with leptospirosis are prepared in the fashion routine for hematological examination and are not fixed prior to the addition of reagent A. It appears, however, that leptospires are more easily found in very thin smears, and thick areas should be avoided. The cells will appear thicker, and spirals are not usually visible due to the presence of serum on the cells. (Fig. 2).

Tissue impression smears have been made by adding a small drop of water to the slide and making the touch preparation through this water. The preparation is air-dried.

The smears are covered by reagent A for 2 to 4 min; they are then rinsed in distilled water. Distilled water and varying concentrations of ammonium hydroxide and ethyl alcohol have been tried, both as rinses and as 10-min washes, with no apparent difference. After the water rinse, reagent B (pH 10.0) is added for about 30 sec. The smears are immediately washed with distilled water, air-dried, and examined under oil immersion.

Leptospires are stained dark-brown to black on a light-to-golden background. Other structures, for example, erythrocytes or other bacteria, stain dark, but the characteristic shape and hooked ends of the leptospires should be sufficient for differentiation from other cells. Also, spirals can often be observed under high-power magnification. The flagella of such organisms as Proteus vulgaris and Vibrio fetus (Fig. 3 and 4) are readily stained if care has been taken to prevent their mechanical removal in the preparation of the smear.

This stain seems to be dependable and is easily
Fig. 1. *Leptospira pomona* in Stuart's broth culture. X1,625.

Fig. 2. *Leptospira canicola* in smear made from heart blood of moribund hamster. X970.

Fig. 3. *Proteus vulgaris*. X970.

Fig. 4. *Vibrio fetus*. X970.

reproduced. The reagents are composed of readily available chemicals. The time involved in staining is minimal, and the smears often show striking cellular detail. No problem with precipitates has been encountered when the pH of reagent B is correct. The technique would seem to have application in both clinical and research laboratories.

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