HISTOCHEMICAL AND IMMUNOHISTOCHEMICAL METHODS FOR DEMONSTRATION OF SPIROCHETES IN SKIN BIOPSIES

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SUMMARY

Spiral or corkscrew-shaped bacteria are classified as spirochetes. They are not stainable with either Gram or Acid fast techniques, commonly used for bacteria. Histochemical stains for the demonstration of spirochetes in routinely processed skin biopsies with suspected borrelial lesions are based on silver stains.

We have tested some filed cases of erythema chronicum migrans and lymphoadenosis benigna cutis employing Warthin-Starry and Steiner & Steiner methods. Spirochetal microorganisms suggesting Lyme Borreliosis have been detected at the dermo-epidermal junction.

Immunohistochemical methods are more specific and sensitive and allow the distinction of Spirochetes in their different subtypes, but require monoclonal antibodies not commercially available for routine procedure.

KEY WORDS

Lyme disease, Borrelia burgdorferi, erythema chronicum migrans, lymphadenosis benigna cutis, monoclonal antibodies, histochemical methods.

The demonstration of spirochetes in tissue specimens is a clinically relevant issue. Some well established histochemical methods (1-9) are available, basically centering upon the impregnation of microorganisms with silver nitrate and subsequent development to a visible metallic deposit. These methods were introduced early this century, especially for the demonstration of *Treponema pallidum* in Syphilis related lesions. However, although these are, time-honored and unquestionably valid techniques, due to their good specificity, underdevelopment is a potential drawback, failing to demonstrate spirochetes.

In addition to false negatives, interference with tissue structures having affinity for silver nitrate and the frequent excessive background staining are sources of difficult interpretation. Recently, treatment of

tissue sections with microwave oven has proven useful in increasing the sensitivity of silver impregnation techniques, also paralleled by a decrease of incubation time and background formation.

Following the recent demonstration (10-12) of a *Borrelia burgdorferi* etiology of Lyme disease (LD), attention has been addressed to argyrophilic techniques in the visualization of spirochetal microorganisms in the skin, especially in geographic areas with a high incidence of LD (13-15). Particularly, in the Trieste area LD is endemic due to ixodes ticks harboring *Borrelia burgdorferi*. The range of clinical manifestation of Lyme disease is broad and includes dermatologic, rheumathologic, neurologic and cardiologic signs. Dermatologic manifestations of borrelial infection are the most frequent and of first set up. The dermatologist, in most cases suspects initially Lyme disease, and requests further corroboration of a diagnosis from the microbiologist, immunologist and histopathologist.

The clinical suspicion of Lyme borreliosis is based on anamnestic criteria, such as living in endemic areas and/or tick bite history in the recent past, and presence of specific dermatologic lesions including erythema chronicum migrans (16-18), lymphadenosis benigna cutis (19-27) acrodermatitis chronica atrophicans (28-31) or other atrophosclerodermical lesions. The direct detection of Borrelia-like microorganisms in tissue with histochemical or immunohistochemical methods is useful for the validation of clinical suspect.

Histochemical staining is relatively easy and inexpensive and may be carried out on the same histologic sections used for routine evaluation.

Immunohistochemical methods are costly, require monoclonal antibodies (not commercially available) and need preferably fresh tissue (32,33).

Histochemical techniques for the demonstration of spirochetes in histologic sections.

Gram stain, modified by Brown-Hopps (34), detects gram positive (blue) and gram negative (red) microorganisms, but is not able to demonstrate spirochetes.

The histochemical detection of Spirochetes in tissue sections is based on the argyrophilia of these microorganisms, which are able to bind silver ions without reducing them to metallic silver.

The main histochemical methods and their recent variants are:

- Warthin-Starry stain (1-9);
- Steiner & Steiner stain (3);
- Dieterle stain (2);
- Churukian-Schenk method (35)

All these techniques are based on silver impregnation of spirochetes and need a developer for reducing the silver to a visible metallic form. Warthin-Starry (WS) method, as modified by Luna (9), has been chosen in our Laboratory as a routine method for the demonstration of spirochetes in tissue sections. Indications to the use WS rather than other methods were: high diagnostic accuracy, availability of reagents, low biological hazards, cost containment, quality of stain, absence of specific background, good contrast between spirochetes and background, stain resistance over time, relatively limited technical difficulty and time requirement.

Recently we have also performed Steiner & Steiner stain (6) in addition to the Warthin-Starry method.

We have tested a series of filed cases, originally diagnosed as erythema chronicum migrans and lymphoadenosis benigna cutis at our Institute during the last few years. These two lesions were found of pertinent interest because of:

1) a relative high frequency of filed histological specimens. The non-specific diagnosis, and the need of ruling out lymphoma was particulary evident for lymphadenosis benigna cutis;
2) early occurrence of skin lesions in respect to the tick bite and high concentration of spirochetes at the biopsy site;
3) importance in recognizing the cause of these lesions for starting the appropriate antibiotic therapy.

In LD lesions, spirochetes were observed at high power (40x-63x dry or 100x at oil immersion) at the dermo-epidermic junction. The microorganisms occurred in small groups or sparse, the microscopic observation of the microorganisms in their entire length requires thorough focusing, with strong accommodative effort for the pathologist.

Warthin-Starry Method

The test is technically not difficult, but it requires a careful execution and takes about two hours. Most of the solutions should be prepared fresh and discared after use. The costs of the chemical reagents is about 8 dollars for each procedure (1 to 10 slides).

Microscopic examinations of slides stained with this method are sometimes difficult due to the presence of silver precipitates, melanin or other dark granules in the background.

The small size of spirochetes requires the microscopic examination at high power. We have modified the Warthin-

Starry stain (as proposed by Luna) introducing sodium thiosulphate for increasing stain stability. Moreover handling of silver nitrate solutions requests the use of gloves and glassware cleared with dichromium acid; plastic forceps are preferable to metallic ones. Sections are cut slightly thicker (3-4 micron) than for routine staining.

- Acidulated water: 0.8 gr citric acid in 500 ml distilled water (DW). Ideal pH of the solution should be 3.8-4.0. Phosphate buffer may used in substitution for citric acid. Refrigerate and use for 1 month.

- 2% silver nitrate: silver nitrate 1 g, in 50 ml of acidulated water.
- 1% silver nitrate (for impregnation): silver nitrate 1 g, in 100 ml of acidulated water.
- 0.15% hydroquinone: Hydroquinone (photographic hydroquinone) in 0,075 g in 50 ml of acidulated water.
- 1% sodium thiosulfate: sodium thiosulfate 1 gr, in 100 ml of DW
- 5% gelatin solution: gelatin 2.5 g in 50 ml of acidulated water.

- DEVELOPING SOLUTION:
silver nitrate 2% solution ... 18 ml
gelatin solution 5% ... 45 ml
hydroquinone solution 0.15% ... 24 ml

use a pre-heated graduated cylinder and mix ingredients in the given order, being sure that the solution is well mixed after each addition. Prepare immediately before use.

Suggested procedure:
- Place the 2% silver nitrate, 5% gelatin, and hydroquinone solutions in separate 50 ml plastic centrifuge tubes. Heat in a water bath at 54°C for at least 1 hour.
- Place a 100 ml graduated cylinder and a chemically cleaned Coplin jar in the oven for at least 1 hour (for developer).
- Deparaffinize and hydrate sections with acidulated water.
- Place slides in the silver nitrate impregnating solution, in a water bath at 43°C for 30 minutes. Do not preheat the solution.
- Immediately prior to use prepare the developer (place in the warm Coplin jar) and place in the 54° C water bath.
- Treat slides with the developer for 3 or 4 minutes. Check after 2 minutes and keep checking frequently until optimal result is obtained.
- Wash slides quickly and throughly in DW.
- Dehydrate sections in 95% and absolute alcohols, and clear in xylene (two changes of each).
- Mount sections with synthetic resin.

Spirochetes stain black on a pale yellow to light brown background (Figure 1).

Steiner & Steiner method

It is not technically a difficult stain. Working solutions should be prepared one day in advance to the use; some may be kept in refrigerator at 4°C and used for one month. Preparing the solutions and performing the staining require about 45 minutes. The cost of the chemical reagents is about 10 dollars for each procedure (1 to 10 slides).

Using (as in our Laboratory) the Swisher-Steiner&Steiner method (modified for microwave oven) it is possible to reduce significantly the background. In spite of this, melanin deposits may interfere with the microscopic evaluation. The demonstration of spirochetes is easier due to the sharp contrast between the spirochetes, stained in black, and the tissue, stained in yellow. Radioactive and toxic hazards of uranile nitrate mandates careful handling (36).

The sections should be thicker than done usually (3-4 micron).

One day before staining prepare:

- 1% uranyl nitrate: uranyl nitrate 1 g in 100 ml DW.
- 1% silver nitrate: silver nitrate 1 g in 100 ml DW. Filter before use. Make fresh each time.
- 0.04% silver nitrate: silver nitrate 0.04 g in 100 ml of DW. Refrigerate and discharge after 1 month.
- 2.5% gum mastic: gum mastic 2.5 g in 100 ml absolute alcohol. Allow gum mastic to dissolve for 24 hr, then filter until clear yellow. Solution may be reused, but should be kept appart from the stock solution. Refrigerate at 4°C.
- 2% hydroquinone: hydroquinone 1 g in 50 ml distilled water. Make fresh each time. (Fresh anhydrous and aqueous hydroquinone should always be used). Do not use anhydrous hydroquinone after 1-2 years.
- reducing solution: mix 10 ml of 2.5% gum mastic, 25 ml of

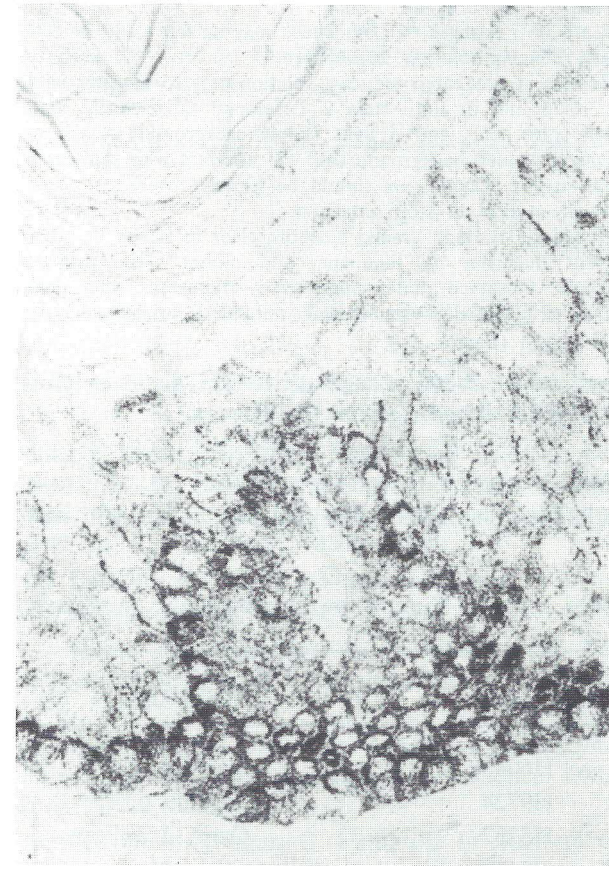

Figure 1. Spirochetes in epidermic basal level of a lymphoadenosis benigna cutis case. Warthin-Starry stain. Original magnification x100.

2.0% hydroquinone, and 5 ml absolute alcohol. Prepare just before each use, filter with 4 filter paper, and add 2.5 ml of 0.04% silver nitrate. Do not filter this silver. Solution will have a milky appearance when the gum mastic is added.

Suggested procedure:
- Deparaffinize and rehydrate tissue with DW
- Enhance stain by placing sections in 1% aqueous uranyl nitrate in room temperature followed by microwave irradiation until boiling point is reached in 25-30 sec. Do not boil. Immediately remove slides to DW. Alternatively, place slides in preheated 1% aqueous uranyl nitrate at 60°C in a water bath for 15 min.
- Rinse in distilled water to prevent cross contamination.
- Place in 1% silver nitrate at room temperature and treat with microwaves until boiling point is reached in 30-45 sec. Do not boil. Remove from oven, loosely cover jar, and allow slides to stand in hot silver nitrate for 5-10 min.

Alternatively, preheat 1% silver nitrate for 20-30 min. in a 60°C water bath, add slides, and allow them to be impregnated at 60°C for 60-90 minutes.

- Rinse in 3 changes of DW.
- Dehydrate in 2 changes of 95% ethyl alcohol.
- Dehydrate in 2 changes of absolute alcohol.
- Treat with 2.5% gum mastic for 5 min.
- Allow to air dray for 1 min.
- Rinse in 3 changes of DW. Slides may be left stand in DW while reducing solution is prepared.
- Reduce in preheated reducing solution in a 45°C water bath for 10-25 min or until stain is developed satisfactorily, with black spirochetes and a light yellow background. Avoid an intensely stained background that will interfere with spirochete identification. Coplin jars should be loosely covered.
- Rinse in DW to stop reduction.
- Dehydrate through graded alcohols, clear in xylene or other solvent that has no limonene, and mount with synthetic medium. Observed under microscope spirochetes stain dark brown to black. Background stains should be bright yellow to light brown (Figure 2).

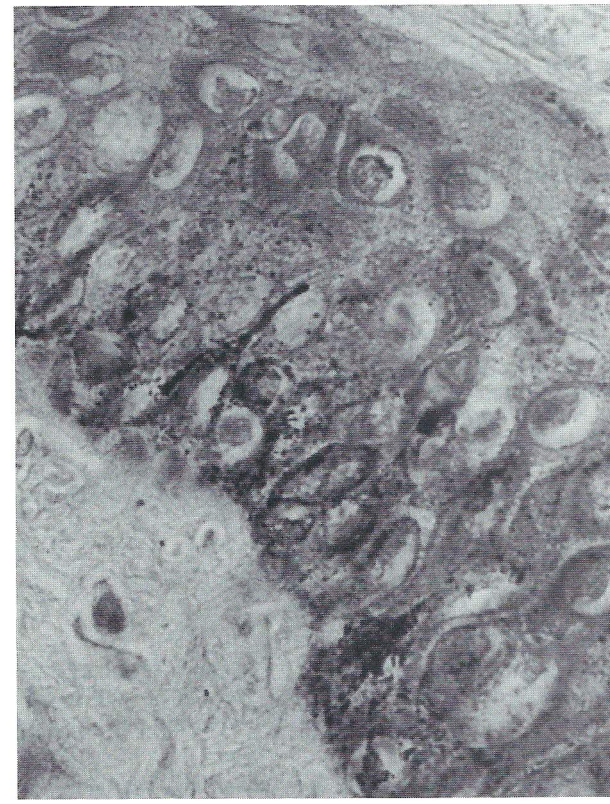

Figure 2. Spirochetes in epidermic level of erythema chronicum migrans case. Steiner & Steiner stain. Original magnification x 100.

Immunohistochemical methods for the demonstration of *Borrelia burgdorferi* in the tissues.

Monoclonal antibodies which recognized specific Borrelial antigens have been developed (32-33). These antibodies (summarized in table I) are specific only for proteins of *Borrelia burgdorferi.* There is no reaction with *Treponema pallidum,* Nichols strain, or with *Leptospira icterohaemorragiae* or other spirocheta-like microorganisms.

These antibodies require fresh frozen tissue sections. Several visualization methods may be used. The long time-consuming systems are based on direct

Table I Some better-known monoclonal antiborrelial antibodies

LABEL	PROTEINs	MOL. WEIGHT (kDal)	SPECIFICITY
H604	flagellin	41	genus
H9724	flagellin	41	genus
H5332	OspA I epitope	31	species
H3TS	OspA II epitope	31	strain
H6831	OspB I epitope	34	strain
H5TS	OspB II epitope	134	strain

fluorescent methods. Indirect methods, e.g. peroxidase antiperoxidase method and avidin biotyn methods are more sensitive but are not standardized for anti-borrelial monoclonal antibodies. In addition fixatives and the histoprocessing critically modify cell antigenic structures adversely affecting immunohistochemical procedures. When immunohistochemical techniques are tested on fixed and embedded tissues, enzymatic predigestion or microwave treatment to unmask antigens are the recommended steps.

Immunohistochemical methods are performed on skin biopsies placed in Michel's transporting medium and snap frozen in liquid nitrogen and cut at 5-6 micron thick. Cryostat sections should be immediately fixed in acetone. Sections are incubated for 30 minutes at room temperature in a humid chamber with primary antibody. The slides are washed for 10 minutes with phosphate-buffered saline solution followed by incubation with 10 fluorescein isothiocyanate conjugated goat antimouse IgG, diluted 1:200 in 1% bovine serum albumin in phosphate-buffered saline solution. The slides are then washed with phosphate-buffer saline solution and examined with a fluorescent microscope. In positive cases, fluorescent deposits are present on papillary dermis and/or epidermal level.

CONCLUSIONS

In our experience, WS and S&S stains are both effective methods in detecting spirochetes in tissues. The microscopic demonstration of spiral bodies with peculiar morphologic structures in dermo-epidermal junction suggests a borrelial origin of the lesion.

Further information, based on the combined histological and serological data, is needed in order to lend support to clinical evidence of LD.

By histochemical and immunohistochemical examinations it is possible to confirm the diagnosis of LD.

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