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Methods for Staining Rickettsia Rickettsii*

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The etiologic agent of Rocky Mountain Spotted Fever is Rickettsia; specifically, Rickettsia rickettsii. The agent was first described in 1909 by Howard Taylor Ricketts, an American pathologist. Dr. Ricketts died in 1910 of typhus fever while investigating that disease.

The Rickettsial microorganisms consist of small pleomorphic rod-like organisms. The organisms have bacterial cell walls, are Gram negative and multiply only inside host cells. The vertebrate host are generally wild mammals, birds, dogs and other small wild animals. The anthropod vectors are ticks. Rocky Mountain Spotted Fever is transmitted to humans by the bite of a tick that has fed on infected rodents, dogs or other small animals.

Rocky Mountain spotted fever is the most severe form of the rickettsial diseases. The onset of symptoms such as chills, fever, malaise and muscular pain begins 2 to 14 days after innoculation by an infected tick. Skin eruptions occur 3 to 5 days after the onset of initial symptoms and usually begins on the ankles and wrists and spreads over the limbs and body. Late in the disease, the eruptions may occur in the oral cavity. The lesions are purpuric, erythematous and pinkish or reddish in color, at first being macules and later petachial. There is enlargement of the spleen, tenderness of the liver and there may be hemorrhagic effusion into the joints. The renal lesions may cause death in uremia. Pneumonia is often the cause of death.

The laboratory diagnosis is usually by analysis of specific serum antibodies with the Weil-Felix test. Smears or paraffin processed tissue may be examined by using fluorescent microscopy, Luna's Giemsa and/or the Pinkerton method.

The purpose of this presentation is to call attention to the staining capability of the two methods cited above for the demonstration of Rickettsiae. The most successful results for paraffin processed tissue can be obtained if tissues are fixed in Zenker's fluid, sectioned at 4 microns and stained with the following methods.

Pinkerton's Method for Rickettsiae

Fixation
Zenker's or Regaud's fluid

Microtomy
Cut paraffin sections at 4 micrometers

Solutions

1% Methylene Blue Solution

Methylene blue	1.0 gm
Distilled water	100.0 ml

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0.25% Basic Fuchsin Solution

Basic fuchsin	0.25 gm
Distilled water	100.0 ml

0.5% Citric Acid Solution

Citric acid	0.5 gm
Distilled water	100.0 ml

Staining Procedure

1. Deparaffinize and hydrate slides to distilled water.
2. Remove mercuric chloride crystals with iodine and clear with sodium thiosulfate. Wash in running water for 5 minutes.
3. Methylene blue solution overnight.
4. Rinse in 95% alcohol for 5 seconds or blue color will be lost.
5. Rinse quickly in distilled water for 2-3 seconds.
6. Basic fuchsin solution for 30 minutes.
7. Decolorize rapidly in citric acid solution for 1-2 seconds, never more than 3 seconds.
8. Continue differentiation in absolute alcohol until nuclei stand out blue and rickettsial organisms red.
9. Clear with xylene, 2 changes.
10. Mount coverslip with resinous media.

Results

Rickettsia (Fig. 1)	bright red
Nuclei	blue

Remarks

This is by far the most reliable method available for demonstrating rickettsiae in paraffin sections. Good differentiation can be controlled since only erythrocytes and rickettsiae are red in well stained sections. All other structures are light blue. Formalin fixation may be used, but results are not as good.

Reference

Simmons, J.S. and Gentzkow, C.F.: Laboratory Methods of the United States Army, 5th ed., Philadelphia, Lea and Febiger, pg. 572, 1944.

Luna's Method for Rickettsiae

Fixation

Zenker's or Regaud's fluid

Microtomy

Cut paraffin sections at 4 micrometers

Solutions

Buffered Water Solution pH 6.8

Buffer salts (pH 6.8)	1.0 gm
Distilled water	1000.0 ml

Stock Giemsa Solution

Giemsa powder	1.0 gm
Glycerin, reagent grade	66.0 ml
Methyl alcohol, acetone free, reagent grade	66.0 ml

Working Giemsa Solution

Giemsa solution (stock)	1.0 ml
Buffered water, pH 6.8	50.0 ml

0.2% Glacial Acetic Acid Solution

Glacial acetic acid	0.2 ml
Distilled water	99.8 ml

Stock Rosin Alcohol Solution

Rosin, white	10.0 gm
Alcohol, 100%	100.0 ml

Working Rosin Alcohol Solution

Rosin solution (stock)	5.0 ml
Alcohol, 95%	40.0 ml

Staining Procedure

1. Deparaffinize and hydrate slides to distilled water.
2. Place in buffer water solution for 60 minutes.
3. Working Giemsa solution overnight.
4. Rinse in buffer water solution.
5. 0.2% glacial acetic acid solution for 1 minute.
6. Rinse in buffer water solution.
7. Differentiate sections individually in working rosin alcohol solution. Check with microscope frequently until rickettsiae appear as violet colored granules (may take up to 3 minutes).
8. Dehydrate in absolute alcohol and clear in xylene, 3 changes each.
9. Mount coverslip with resinous media.

Results

Rickettsia (Fig. 2)	violet
Nuclei	blue
Cytoplasm, connective tissue	pink
Erythrocytes	salmon

Remarks

The differentiation at step 7 is most critical and only if this is properly performed can one expect good results.

Formalin fixed tissue can be used, but results are not as good as suggested fixatives. The rosin used for treating violin strings can be used for making the alcoholic rosin solution.

*** Note**

Although the organisms of Rickettsia Rickettsii (Fig 1 and 2) are shown, other Rickettsial organisms will stain in a similar fashion with the suggested stains.

Reference

Saunder's Dictionary and Encyclopedia of Laboratory Medicine and Technology. James L. Bennington, editor. W.B. Saunders Co., Philadelphia, PA.

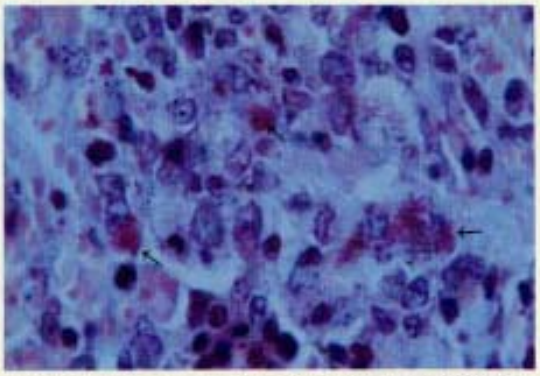

Figure 1. Demostrates masses (arrows) of pleomorphic organisms of the Rickettsia rickettsii type. Note the good differentiation of Rickettsia from the surrounding tissue. Pinkerton's method, X 1000.

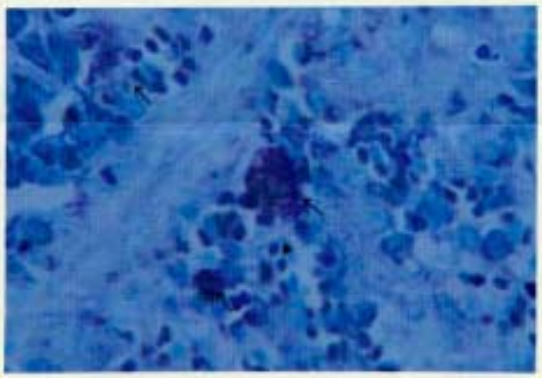

Figure 2. Demonstrates the same organisms (arrows) except that they were stained with Luna's method, X 1000.

Improved Preparation of Harris' Hematoxylin

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The popularity of Harris' hematoxylin in histology laboratories cannot be overstated. A modification eliminating alcohol as the solvent has been described by Hall.[1]

The idea of not dissolving the hematoxylin crystals in alcohol was intriguing. It was found, however, that boiling again after the addition of mercuric oxide, the solution could "curdle". Apart from that, there is always the likelihood of "spills" as well as the potential fire hazard. To obviate this, a safer and simpler preparation requiring no boiling has been developed and is presented here in the hope that other laboratories may find it useful.

Debidin's Modification of Harris' Hematoxylin

Hematoxylin	2.5	gm
Potassium or ammonium alum	50.0	gm
Distilled water	500.0	ml
Mercuric oxide (red)	1.25	gm
Glacial acetic acid, 2%	10.0	ml

Dissolve the potassium or ammonium alum in distilled water in a conical flask (preferably with the aid of a magnetic stirrer). Add the hematoxylin. Cover the flask and leave mixture in a 60° C oven overnight. Remove from oven, add mercuric oxide slowly and stir until cold. Filter and add glacial acetic acid. The solution is ready for use.

Remarks

Adding the glacial acetic acid before use gives a more precise and selective staining. It is advisable to prepare small quantities each month, rather than a large stock. Nuclear staining with this hematoxylin is sharp and reproducible.

The advantages of this method of preparation of Harris' hematoxylin are:

1. It is easier to prepare.
2. It is less time consuming.
3. It is a tidy operation.
4. Less likelihood of "spills" as well as a potential fire hazard.

Reference

1. Carter, D.W.: Histo-Logic, Vol. X, No. 2, 1980.

Modified Method II for Pancreatic Beta Cell Granules

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The method provided below is a modification which has proven very useful and reproducible in the author's hands. The changes introduced in this method are discussed at the point where they are made, or under remarks.

This aldehyde fuchsin procedure can be used to stain all other entities normally stained by aldehyde fuchsin; elastic fibers, hepatitis B surface antigens, mast cells and pancreatic beta cell granules.

Fixation
10% buffered neutral formalin.

Microtomy
Cut paraffin sections at 6 micrometers.

Solutions

Wenger's Aldehyde Fuchsin Solution
This modified aldehyde fuchsin solution is offered as an alternate to those laboratories that may face a problem purchasing paraldehyde for use in the Gomori's aldehyde fuchsin solution.

Basic fuchsin	2.5 gm
Alcohol, ethyl 70%*	500.0 ml
Hydrochloric acid, conc.	5.0 ml
Potassium dichromate	5.0 gm

Combine the above ingredients, cap the container tightly and allow to stand at room temperature for 5 days. Check solution by smelling to ensure formation of acetalaldehyde. Acetalaldehyde can be detected by its characteristic pungent fruity odor. If acetalaldehyde is not detected within 24 hours, ethyl alcohol and/or potassium dichromate was not added, or possibly an insufficient amount of hydrochloric acid was used. At the end of the 5 day period, check the quality of the solution by staining an elastin control. If elastic tissue fibers are well demonstrated, cap the bottle tightly and refrigerate the solution until ready to use.

0.25% Metanil Yellow

Metanil yellow	0.25 gm
Distilled water	100.0 ml
Acetic acid, glacial	2 drops

Thiosulfation Solution A (Stock)
(0.0125 M cupric sulfate complexed with 0.25 M Tris, [hydroxymethyl] aminomethane)

Cupric sulfate penta hydrate	1.56 gm
Tris (hydroxymethyl) aminomethane	15.14 gm
Distilled water	q.s. to 500.0 ml

Adjust to pH 9.0 if necessary, with 1% aqueous sodium hydroxide. This solution is stable at room temperature.

Thiosulfation Solution B (Stock)
(0.2 M sodium metabisulfite)

Make Fresh

Sodium metabisulfite	3.8 gm
Distilled water	100.0 ml

This solution is stable for several days.

Thiosulfation Solution (Working)

Solution A (stock)	80.0 ml
Solution B (stock)	20.0 ml

This solution is stable for up to 2 days.

Kernechtrot (Nuclear Fast Red)
Dissolve 0.1 gm of Kernechtrot (nuclear fast red) in 100 ml of a 5% solution of aluminum sulfate, with the aid of heat. Cool, filter, add a gram of thymol as a preservative.

Staining Procedure

1. Decerate slides and hydrate to distilled water.
2. Place slides in fresh thiosulfation working solution for 3 hours.
3. Wash slides in tap water for 1 minute and rinse in distilled water.
4. Rinse slides in 95% alcohol.
5. Place slides in Wenger's aldehyde fuchsin solution for 2 minutes.
6. Rinse slides in 95% alcohol until no more stain runs off the slides. See note below.
7. Stain slides in metanil yellow for 30 seconds.
8. Rinse slides in distilled water.
9. Stain in Kernechtrot for 3 minutes.
10. Rinse in 5 changes of distilled water.
11. Dehydrate in 95% alcohol, absolute alcohol and clear in xylene, 2 changes each.
12. Mount coverglass with resinous media.

Results

Beta cell granules	purple
Background	yellow
Nuclei	red

Remarks
*Ethyl alcohol reacts with potassium dichromate to form acetaldehyde[1]. This procedure has been modified from the original published by Bussolati and Bassa[2]. The major modification is the substitution of Tris (hydroxymethyl) aminomethane for ammonium hydroxide, which adds stability to stock solution A, thus prolonging the shelf life of the solution.

Note
Check control slide microscopically. If beta cells are inadequately stained, repeat steps 2 through 6.

References

1. Merck Index, 8th ed., Merck & Co., Rahway, NJ, p. 4, 1968.
2. Bussolati, G. and Bussa, T.: Thiosulfation Aldehyde Fuchsin (TAF) Procedure for the Staining of Pancreatic B Cells. Stain Tech., 49: No. 5, p. 313, 1974.

Campbell's Polychrome Stain – New Use In Histology

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Fixation
10% neutral buffered formalin.

Embedding
Cut paraffin section at 6 micrometers

Solutions

Campbell's Polychrome Stain

Toluidine blue	1.0 gm
Methylene blue	1.0 gm
Azure A	0.25 gm
Giemsa powder	0.25 gm
80% Ethyl alcohol	650.0 ml

Combine the dyes with the 80% alcohol. Dissolve with the aid of gentle heat; allow to cool and filter. Stain is now ready for use. The stain is stable at room temperature and keeps indefinitely.

1% Acetic Acid

Glacial acetic acid	1.0 ml
Distilled water	100.0 ml

Staining Procedure
1. Deparaffinize slides through 2 changes of xylene, absolute and 95% alcohol, to distilled water, as usual.
2. Stain in Campbell's polychrome stain for 10 to 20 minutes.
3. Rinse in distilled water.
4. 1% acetic acid solution, 2 dips.
5. 100% alcohol, 2 dips.
6. Ethyl acetate, 2 dips.
7. Clear in xylene and mount coverslip with resinous media.

Results
The common characteristics of polychrome staining reaction. This method is very satisfactory for central nervous system tissue and for mast cells which stain red.

Reference

Campbell, J. and Saeger, K.: A New Polychrome Stain. Histo-Logic, Vol. IX, No. 3, 1979.

Reasons for Sections Falling Off of Slides

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During performance of staining procedures, a problem encountered by many laboratories is tissue sections falling off the glass slide. There are many reasons for this problem; some of the more common are presented herein.

Inadequate Adhesion
Inadequate section adhesion often results from improper application of adhesives. For example, if one utilized granular gelatin by sprinkling it upon the surface of the floatation bath, one may remove an excessive amount during the surface cleaning of the bath by the use of a paper towel. This is particularly true if the gelatin is not mixed adequately with the water.

Section Over-Expansion
Sections frequently fall off slides due to section over-expansion on the surface of the floatation bath. This occurs when the water temperature is excessive; 45° C or over. In this case, the section expands beyond its normal size and is picked up on a slide in this extended position. During the drying and/or staining steps, the section attempts to shrink to its original shape. During this process, the bond between the adhesive and the section are broken at microscopic levels, allowing various liquids to seep between the section and the slide. This water reacts with the adhesive and breaks the bonds further, allowing the section to detach.

Alkaline Solutions
Protein adhesive bonds between the section and the glass slide are broken when slides are exposed to alkaline solutions; i.e., ammoniacal silver, H&E bluing solutions, etc. Conversely, the bonds of protein adhesive do not break easily in acid solutions.

Glycerin-Egg Albumin
The glycerin used in compounding Mayer's egg albumin acts as a stabilizer keeping the egg albumin (protein) in a liquid state. If one does not allow the glycerin to dissolve adequately in the floatation bath, the protein will not gel or set adequately when the slides are dried on the slide warming table or oven. The protein will remain dissolved in residual glycerin, thereby having no adhesive qualities. This will allow the sections to become detached from the slide during subsequent staining.

Enzyme Treatment
Any section treated with an enzyme may fall off if not properly adhered to the slide. Examples of enzyme treatments are: Diastase in the PAS technique; hyaluronidase in the procedures for mucosubstances; trypsin or other enzymes for immunochemical procedures.

Ninth Annual Practical Stain-Technology "Wet" Workshop and Seminar March 20-25, 1988

Presented by: Lee G. Luna, HT (ASCP)
The Center for Histotechnology Training

This five day extensive wet workshop and seminar will afford the registrants the opportunity to perform *25 special stains, two microwave and two immunochemical staining procedures demonstrating more than 35 pathologic entities.*

Some of the entities which will be stained are: Gram positive and Gram negative bacteria; hepatitis B surface antigen; inclusion bodies; muscle; insulin; all pathogenic fungi; calcium; glycosaminoglycans (mucosubstances); rabies; myelin sheath; nerve fibers; mast cells; lepra and tubercle bacilli; nucleic acids - RNA and DNA; amyloid; silver reactive cell granules from the neuroendocrine system; copper; reticulum and other connective tissues; cross striations in rhabdomyosarcoma; fibrin; spirochetes; Rickettsiae; Legionnaires disease bacilli; cat scratch fever; and S-100 protein. Also, the proper tinctorial qualities of a good hematoxylin and eosin (H&E) will be discussed in great detail.

In addition to the practical special staining aspects, lectures will be presented daily (to include evenings) on Chemistry of Staining; Fixation; Staining Mechanisms; Tissue Identification for Histotechnologists; Microtomy; Preferred Controls; Introduction to Immunochemistry Staining; Decalcification and various other subjects directly related to the production of high quality microscopic slides. National Society for Histotechnology Continuing Education Units (2.3) will be awarded.

For program and related information, contact:
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(301) 330-1200

Correction

Histo-Logic 17: No 1, 235, 1987 contained an article entitled "The Warthin-Starry Impregnation Technique with the Microwave Oven" by Kathy Vail. This procedure had limited usuage in our laboratories. Although the Dieterle Silver Impregnation and Warthin-Starry procedures with and without the microwave modifications were used in our laboratories in the past, we now use exclusively the modified Steiner procedure for staining the microorganisms discussed in the Vail article. This Steiner technique was reported by W. Garvey, et al., in the Journal of Histotechnology 8: 15-17, 1985

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The editor wishes to solicit information, questions, and articles relating to histotechnology. Submit these to: Lee G. Luna, Editor, Histo-Logic, P.O. Box 36, Lanham, Maryland 20706. Articles, photographs, etc., will not be returned unless requested in writing when they are submitted.