A Reliable Silver Staining Method for Identification of *Pneumocystis carinii* in Histologic Sections

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SENBA, M. A Reliable Silver Staining Method for Identification of *Pneumocystis carinii* in Histologic Sections. Tohoku J. exp. Med., 1984, 143 (4), 397-404 —— The acquired immune deficiency syndrome (AIDS) is characterized by the development of Kaposi's sarcoma and several opportunistic infections including pneumonia caused by *Pneumocystis carinii*. As a staining method for *Pneumocystis carinii*, the methenamine-silver nitrate method has been used routinely. Most of fungi can be stained easily by this technique but the identification of *Pneumocystis carinii* is not always easy. The author has improved the method by using ammoniacal silver nitrate and found that this was a reliable staining method for *Pneumocystis carinii*. Moreover, this method proved to be superior to others in demonstrating *Pneumocystis carinii* in histologic sections. —— *Pneumocystis carinii* ; staining method.

Of 2,008 cases of AIDS in the United States of America reported to the Centers for Disease Control (CDC) between June 1981 and August 8, 1983, the most common “marker” diseases have been *Pneumocystis carinii* pneumonia (1,016), Kaposi’s sarcoma (533), or both (148) (Curran 1983). Therefore, a reliable staining method for *Pneumocystis carinii* is extremely useful in diagnosis of AIDS.

Many methods have been developed for the purpose of staining *Pneumocystis carinii* in paraffin sections, including Gomori’s methenamine-silver nitrate (Gomori 1946), Grocott’s methenamine-silver nitrate (Grocott 1955), modified Grocott’s methenamine-silver nitrate (Smith and Hughes 1972; Churukian and Schenk 1977; Mahan and Sale 1978; Pintozzi 1978; Musto et al. 1982), toluidine blue O (Chalvardjian and Grawe 1963), cresyl echt violet (Bowling et al. 1973), and ammoniacal silver nitrate (Senba 1983).

Among these staining methods, the most popular is the methenamine-silver nitrate method, which can stain fungi easily but is difficult to stain *Pneumocystis carinii* constantly and to obtain stable results. However, it has been found that constant and satisfactory results can be obtained by modifying the silver impregnation method (Luna 1960; Lillie and Fullmer 1975). The author made a preliminary report on a new modification (Senba 1983).
**MATERIALS AND METHOD**

The lung specimens from five autopsy cases of *Pneumocystis carinii* infection at Nagasaki University Medical School were used (Table 1). The material was fixed in formalin and embedded in paraffin.

The steps involved in the modified silver staining method for *Pneumocystis carinii* are as follows: (1) Deparaffinize and hydrate in distilled water. (2) Treat with oxidizing solution for 2–3 min. Oxidizing solution: Dissolve 0.3 g of potassium permanganate in 100 ml of distilled water and add 0.3 ml of sulfuric acid or 0.6 ml of hydrochloric acid. (3) Wash in running water. (4) Treat with 2% oxalic acid solution until sections become clear. (5) Wash in running water. (6) Treat with 2% ferric ammonium sulfate solution for 1 min. (7) Wash in running water. (8) Treat with ammoniacal silver nitrate solution for 5 min. Ammoniacal silver solution: To 20 ml of 10% silver nitrate solution add 0.4 g of sodium hydroxide, and then dropwise 28% ammoniacal hydroxide, until only a few granules of the resulting precipitate remain on the bottom of the cylinder. And add distilled water to make 100 ml. Dilute 1 part of ammoniacal silver nitrate solution with 4 parts of distilled water for use. Store in the refrigerator and use as needed. (9) Wash in distilled water for 30 sec. (10) Treat with 95% alcohol solution for 1 sec. (11) Treat with 5% formalin for 1 min. (12) Wash in running water. (13) Treat with 0.5% gold chloride for 5 min. (14) Wash in running water. (15) Treat with 2% oxalic acid solution for 5 min. (16) Wash in running water. (17) Treat with 10% sodium thiosulfate solution for 5 min. (18) Treat with 0.2% acetic solution for 1 min. (19) Wash in running water. (20) Treat with nuclear fast red (Kernechtrot) solution for 5 min. Nuclear fast red solution: Dissolve 0.1 g of nuclear fast red in 100 ml of a solution of aluminum sulfate with aid of heat. Cool and filter. (21) Wash in running water. (22) Dehydrate, clear and mount.

**RESULTS**

*Pneumocystis carinii* was stained black (Figs. 2, 3a, 4 and 5) by this ammoniacal silver nitrate method. With silver particles, the ammoniacal silver nitrate method did not stain nuclei, but the methenamine-silver nitrate method stained many nuclei including those of leukocytes. By the ammoniacal silver nitrate method, nuclei were stained red with nuclear fast red. Moreover, the methenamine-silver nitrate method also stained periodic acid-Schiff (PAS) positive materials associated with *Pneumocystis carinii* (Fig. 3b), but the ammoniacal silver nitrate method did not stain these materials (Fig. 3a). Fungi were stained

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**Table 1. Cases of Pneumocystis carinii pneumonia**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Pathological diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>51</td>
<td>M</td>
<td>Rhabdomyosarcoma</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>F</td>
<td>Adult T cell leukemia</td>
</tr>
<tr>
<td>3</td>
<td>51</td>
<td>F</td>
<td>Adult T cell lukemia</td>
</tr>
<tr>
<td>4</td>
<td>67</td>
<td>M</td>
<td>Lung carcinoma</td>
</tr>
<tr>
<td>5</td>
<td>76</td>
<td>M</td>
<td>Lung carcinoma</td>
</tr>
</tbody>
</table>

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Fig. 1. Case 3. Lung section showing interstitial pneumonitis and typical honey-combed material in the alveolar spaces. Hematoxylin and eosin stain. Original magnification ×200.

Fig. 2. Case 3. Numerous cysts of Pneumocystis carinii in the alveolar space. Note the typical round, oval and crescent forms. Ammoniacal silver nitrate method. Original magnification ×200.
Fig. 3. a: Case 3. Without nuclear staining. Excellent results have been obtained in illustrating cysts of *Pneumocystis carinii*. Ammoniacal silver nitrate method. Original magnification ×400.

b: Case 3. Without nuclear staining. This staining shows many nuclei including those of leukocytes in addition to *Pneumocystis carinii*. PAS positive materials are also stained. Methenamine-silver nitrate method. Original magnification ×400.
Fig. 4. Case 1. Cysts of *Pneumocystis carinii* (left) and fungi (right) are seen in the same lung section. Ammoniacal silver nitrate method. Original magnification × 400.

Fig. 5. Case 2. Cysts of *Pneumocystis carinii* are seen in the alveolar macrophage (arrow). Ammoniacal silver nitrate method. Original magnification ×400.
Fig. 6. a: Arrows indicate cryptococci in the alveolar space. Ammoniacal silver nitrate method. Original magnification $\times 400$.
b: Arrows indicate cryptococci in the alveolar space. Methenamine-silver nitrate method. Original magnification $\times 400$.

Fig. 7. Reticulin fibers present in the spleen. Nuclear staining procedure is omitted in Fig. 7a. This procedure does not allow to stain the cytoplasm with silver particles and reticulin fibers show up. Ammoniacal silver nitrate method. Original magnification $\times 200$. 
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black (Fig. 4). Cryptococci were stained black but their shape was different from those stained by the methenamine-silver nitrate method (Figs. 6a, b). Reticulin fibers were stained black but the cytoplasm was not stained with silver particles (Fig. 7a–8b).

DISCUSSION

In staining Pneumocystis carinii by the methenamine-silver nitrate method, it is quite difficult to obtain stable and satisfactory results. The author modified the silver impregnation method by adding hydrochloric acid or sulfuric acid to potassium permanganate solution which had been used as the oxidizing solution. By this procedure, silver particles adhering to the cytoplasm could be removed. As a result, the staining of Pneumocystis carinii with good contrast has become constantly available. This is due to strong oxidating power of potassium permanganate in dilute hydrochloric acid or sulfuric acid.

In the silver impregnation method, paraffin sections often detached from the slides when they were washed after treatment with sodium thiosulfate solution. However, this problem has been solved by rinsing in 0.2% acetic acid after the sodium thiosulfate solution treatment. The nuclei were stained well due to a raise in the concentration of sodium thiosulfate.

Acknowledgments

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References


Fig. 8. Reticulin fibers present in the liver. Nuclear staining procedure is omitted in Fig. 8a. This procedure does not allow to stain the cytoplasm with silver particles. Ammoniacal silver nitrate method. Original magnification ×200.


