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New Grocott Stain without Using Chromic Acid

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We established a new "ecological" Grocott stain for demonstrating fungi, based upon a 4R principle of refusal, reduction, reuse, and recycle of waste management. Conventional Grocott stain employs environmentally harsh 5% chromic acid for oxidization. Initially, we succeeded in reducing the concentration of chromic acid from 5% to 1% by incubating the solution at 60°C and using five-fold diluted chromic acid solution at which point it was reusable. Eventually, we reached the refusal level where 1% periodic acid oxidization was efficient enough, when combined with preheating of sections in the electric jar, microwave oven, or pressure pan. For convenience sake, we recommend pressure pan heating in tap water for 10 min. Stainability of fungi in candidiasis and aspergillosis was comparable with conventional Grocott stain, while *Mucor* hyphae showed enhanced staining. The modified sequence was further applicable to detecting a variety of mycotic pathogens in paraffin sections. Our environmentally-friendly Grocott stain also has the advantage of avoiding risk of human exposure to hexavalent chromium solution in the histopathology laboratory. The simple stain sequence is can be easily applied worldwide.

Key words: new Grocott stain, chromic acid, periodic acid, refusal, waste management

I. Introduction

Periodic acid-Schiff (PAS) reaction [13], Gridley stain [5], and Grocott stain [6] represent histochemical techniques for demonstrating fungi in tissue sections. Of these three techniques, Grocott stain shows the highest sensitivity for detecting fungi and other polysaccharide-rich microorganisms in routinely prepared paraffin sections. Gomori's methenamine-silver nitrate [4] and chromic acid comprise the major reagents used in conventional Grocott stain.

The staining sequence for Grocott stain has remained unchanged for more than half a century. Chromic acid (H_2CrO_4) contains hexavalent chromium and is capable of oxidizing many kinds of organic compounds, but it is highly corrosive, toxic, and carcinogenic to the lung [22]. At present, chromic acid oxidation is never used on an industrial scale. When it is disposed of, hexavalent chromium in the chromic acid solution must be reduced to non-hazardous trivalent chromium. It is actually impossible for medical laboratory staff to avoid the chemical hazard to environment and human body posed by this chemical, if we continue to use it. We believe that it is time to stop using this hazardous chemical for staining in order to immediately improve such environmentally compromising working conditions.

A strategy for curbing the environmental impact of waste is simple, and represented by the 4R principle of refusal, reduction, reuse, and recycle [17]. In order to develop an environmentally-friendly or "ecological" modification of Grocott stain, we firstly tried to reduce and reuse/recycle and finally to refuse chromic acid solution.

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II. Materials and Methods

Samples

A total of nine fungal lesions (candidiasis 3, aspergillosis 3, and mucormycosis 3) were obtained from the autopsy file in Fujita Health University Hospital, Toyoake, Japan, and were used for the basic analysis. A variety of mycotic infections were further evaluated with our final modification protocol (Grocott stain without using chromic acid). These included pneumocystosis 3 lesions, cryptococcosis 3, histoplasmosis 2, penicilliosis marneffei 1, chromomycosis 2, dermatophytosis 2, chlorella infection 1, and actinomycosis 3. All the tissues were routinely fixed with 10% formalin and embedded in paraffin wax. The presence of fungi in each section was confirmed with hematoxylin and eosin stain, PAS reaction, and conventional Grocott stain.

Dilution of chromic acid for "reduction and reuse/recycle"

Conventional Grocott stain employs 5% chromic acid solution, incubated at room temperature for 1 hr. The concentration of chromic acid was reduced to 1%. The incubation was set at 60°C for 1 hr, and the 1% chromic acid solution kept at room temperature was used repeatedly over the period tested.

Oxidization with periodic acid for "refusal" of chromic acid

The oxidization step was changed from chromic acid to 1% (ortho) periodic acid (H_5IO_6). Firstly, sections were oxidized in 1% periodic acid for 1 hr, 3 hr, 6 hr, 12 hr and 24 hr at room temperature, at 37°C or at 60°C. Secondly, sections were heat-treated by employing 4 different conditions: incubator warming at 60°C for 6 hr, electric jar boiling at 95°C for 30 min, 500 watt microwaving at 100°C for 10 min, and boiling in a pressure pan (T-fal, Rumily, France) at >120°C for 10 min. As soaking solutions, tap water, distilled water, 10 mM citrate buffer, pH 6, and 1 mM ethylenediamine tetraacetic acid (EDTA) solution, pH 8, were chosen. Subsequently, sections were oxidized with 1% periodic acid for 10 to 60 min at room temperature. In some experiments, sections were heated after periodate oxidation for comparison.

Ethical issue

The present study was approved by the institutional ethical review board for clinical and epidemiological investigations at Fujita Health University, Toyoake.

III. Results

Use of diluted chromic acid solution

Oxidization with 60°C-warmed 1% chromic acid for 1 hr gave a satisfactory result comparable with conventional Grocott stain (Fig. 1). Nonspecific co-staining in the connective tissue component occasionally seen in conventional Grocott stain was suppressed by warm incubation with the diluted chromic acid. Repeated use (reuse and/or recycle) of 1% chromic acid solution, stored at room temperature,

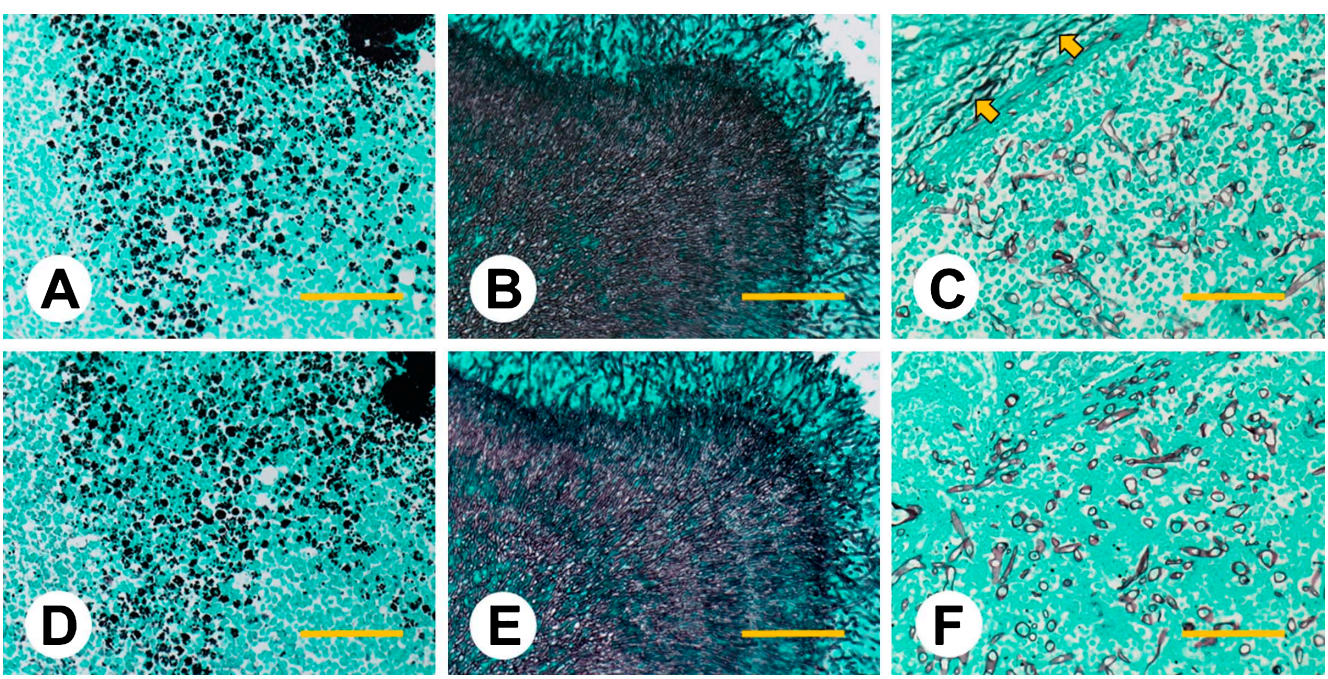

Fig. 1. Grocott stain with reduced concentration of chromic acid. (**A** & **D**) candidiasis, (**B** & **E**) aspergillosis, and (**C** & **F**) mucormycosis. (**A, B, C**): Conventional Grocott stain with 5% chromic acid oxidation at room temperature for 1 hr. (**D, E, F**): Modified Grocott stain with 1% chromic acid oxidation at 60°C for 1 hr. Modified Grocott stain with 1% chromic acid solution gave satisfactory staining comparable with the conventional method. Co-staining in the vascular connective tissue component seen in the conventional method in panel **C** (arrows) disappeared in the modified sequence (panel **F**). Bars=50 μm.

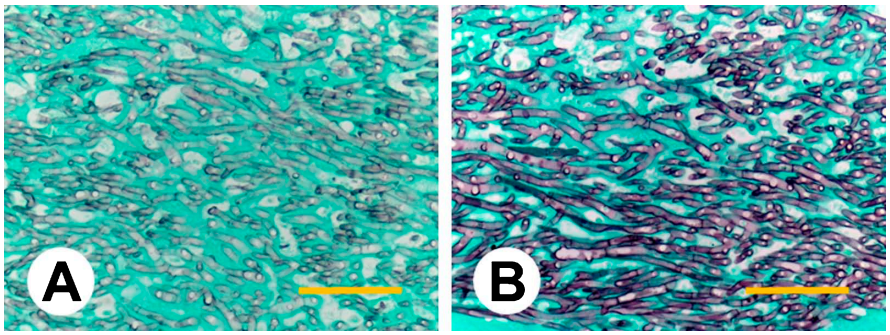

Fig. 2. Effect of preheating in demonstrating *Aspergillus* in lung. Periodic acid oxidation was performed before (**A**) and after (**B**) heating in tap water in an electric jar. Effect of heat pretreatment is evident. Bars=50 µm.

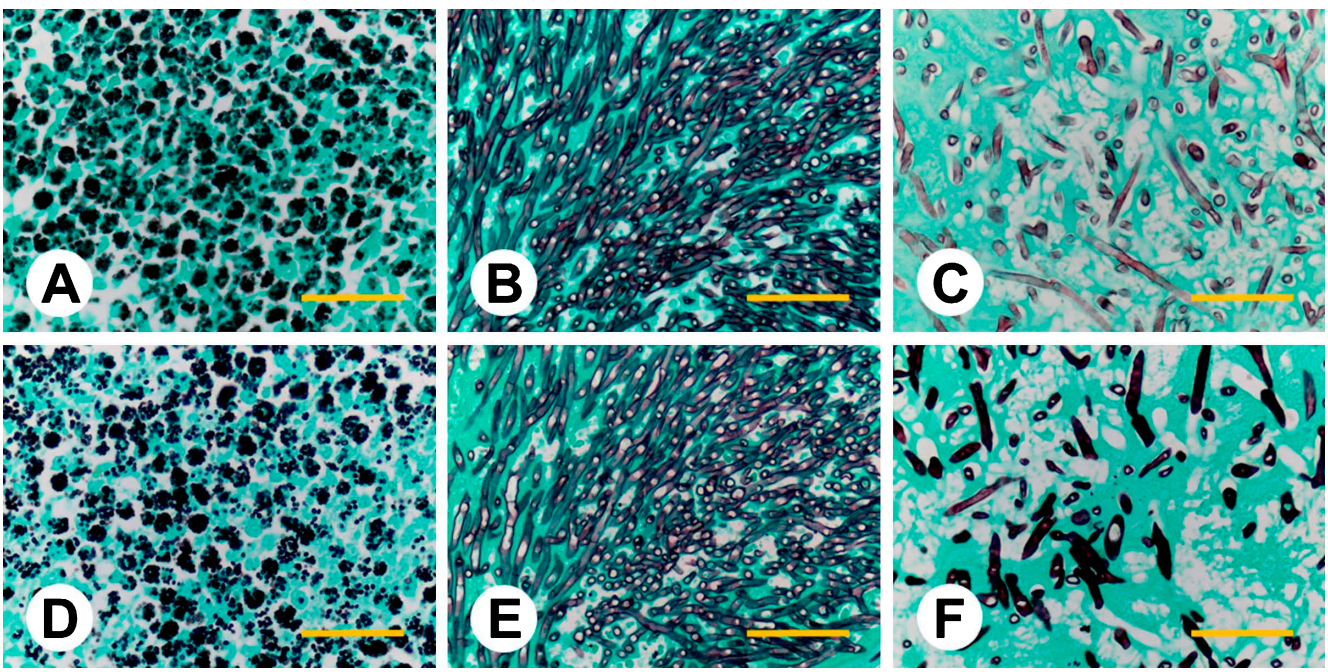

Fig. 3. Effect of preheating in demonstrating fungi in lung with modified Grocott stain employing 1% periodic acid oxidation at room temperature for 10 min. (**A** & **D**) candidiasis, (**B** & **E**) aspergillosis, and (**C** & **F**) mucormycosis. (**A–C**): Conventional Grocott stain using 5% chromic acid solution. (**D–F**): Pressure pan heating in tap water for 10 min. Both conditions gave distinct staining for *Candida albicans* and *Aspergillus fumigatus*. *Mucor* hyphae showed enhanced staining in our modified technique (panel **F**), when compared with conventional Grocott stain (panel **C**). Bars=50 µm.

was possible up to 4 weeks. Actually, we reused/recycled the solution once a week for a total of four times.

Use of 1% periodic acid solution

Treatment with 1% periodic acid solution at room temperature showed weaker staining even when the incubation period was prolonged for 24 hr. Staining intensity showed a peak at 6 hr oxidization in 1% periodic acid solution, but the condition was unsatisfactory and not so stable. Periodic acid solution preheated at 37°C or at 60°C failed to improve the stainability.

Heat pretreatment of deparaffinized sections was effective for enhancing the oxidizing effect in 1% periodic acid solution. All 4 types of soaking solution (tap water, distilled water, 10 mM citrate buffer, pH 6, and 1 mM EDTA, pH 8) provided comparable effects. Figure 2 demonstrates the effect of electric jar preheating in tap water at 95°C for 30 min. Heating after periodate oxidation was ineffective. After preheating sections, oxidation in 1% periodic acid at room temperature for 10 min gave a staining intensity comparable with conventional Grocott stain. Heat pretreatment in electric jar, microwave oven and pressure pan all gave satisfactory staining for candidiasis and aspergillosis (Fig. 3). Little tissue damage was observed after heating. When preheated in an incubator at 60°C for 6 hr, 1% periodic acid oxidation accelerated co-staining in connective tissue components. It should be noted that the staining intensity of hyphae in mucormycosis was significantly enhanced in our "ecological" modification of Grocott stain, while conventional Grocott stain resulted in weak staining (Fig. 3C).

Figure 4 demonstrates the application of our modi-

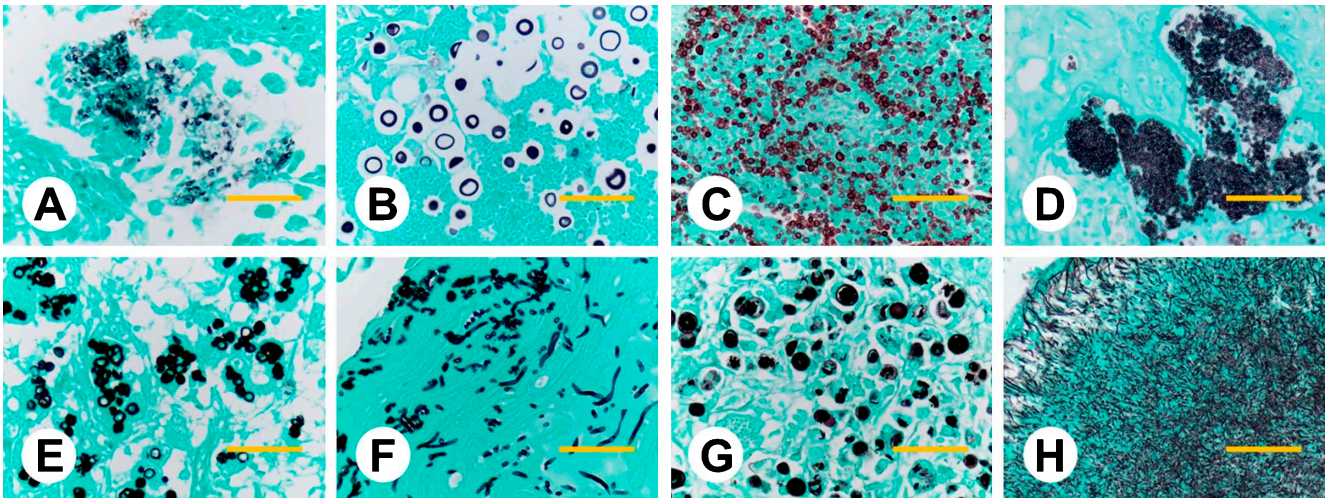

Fig. 4. Demonstration of mycotic pathogens in biopsy specimens with our modified Grocott stain avoiding the use of chromic acid. (**A**) Pulmonary pneumocystosis in AIDS, (**B**) pulmonary cryptococcosis in AIDS, (**C**) pulmonary histoplasmosis, (**D**) cutaneous penicilliosis marneffei in AIDS, (**E**) cutaneous chromomycosis in hemodialysis, (**F**) dermatophytosis, (**G**) cutaneous chlorella infection in bharal (Himalayan blue sheep), and (**H**) pharyngeal actiomycosis. Both yeast type and hypha-forming fungi (**A–F**), as well as chlorella (**G**) and filamentous bacteria (**H**), are stained black with our ecological sequence. Bars=50 μm.

Table 1 *Procedures for the new Grocott stain*

1) Deparaffinize sections in xylene and soak them in alcohol.
2) Rise sections in running tap water.
3) Heat sections in tap water using either electric jar at 95°C for 30 min, 500 watt microwave for 10 min or pressure pan for 10 min.
4) Cool down sections for 15 min on a table and then rinse them in running tap water.
5) Keep methenamine-silver nitrate solution in a 60°C oven.
6) Incubate sections in 1% periodic acid solution at room temperature for 10 min.
7) Rinse sections in running tap water and then in distilled water (3 changes).
8) Soak sections in the methenamine-silver nitrate solution pre-heated at 60°C for 40 to 60 min.
9) Rinse sections in distilled water (3 changes).
10) Replace silver with 0.1% gold chloride acid tetrahydrate ($HAuCl_4 \cdot 4H_2O$) at room temperature for 3 min.
11) Rinse sections briefly in distilled water.
12) Fix sections in 2% sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) at room temperature for 3 min.
13) Rise sections in running tap water.
14) Counterstain sections in 0.04% light green solution at room temperature for 5 min.
15) Rise sections in running tap water.
16) Dehydrate sections in ethanol, change in xylene and mount with a cover glass.

[Preparation of methenamine silver solution]
Prepare the methenamine-silver nitrate solution just before use. Add 5 ml of 5% silver nitrate stock solution ($AgNO_3$) to 100 ml of 3% methenamine (hexamethylenetetramine) stock solution ($(CH_2)_6N_4$). Then, add 25 ml of distilled water and 4 ml of 5% sodium borate stock solution ($Na_2B_4O_7 \cdot 10H_2O$) to 25 ml of mixture solution. Finally, mix 2–3 drops of 5% gelatin to avoid the silver mirror reaction.

fication without using chromic acid to a variety of mycotic pathogens in paraffin sections. Clear black staining was obtained in all the additionally evaluated fungal and related pathogens, such as *Pneumocystis jiroveci*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Penicillium marneffei*, chromomycotic fungi, *Dermatophytes*, *Chlorella*, and *Actinomyces israelii*.

Table 1 summarizes the staining sequence of our new ecological modification of Grocott stain, employing preheating of sections in tap water and oxidation in 1% periodic acid solution. For preheating, either the electric jar, microwave oven or pressure pan is available, but we recommend pressure pan heating for 10 min for convenience sake.

IV. Discussion

We established a newly modified Grocott stain technique that does not use chromic acid. Our environmentally-friendly method consistently yielded clear and satisfactory results for black staining of fungi. So far, several modifications of Grocott stain have been reported [14–16, 19, 21]. To the best of our knowledge, no research was focused on the ecological point of view eliminating the use of chromic acid.

Periodic acid is a common oxidative reagent widely employed in PAS reaction [13] and periodic acid methenamine-silver (PAM) stain [11], and is also used for quenching endogenous peroxidase in immunostaining [9].

It is well known that periodic acid oxidizes vicinal diol hydroxyl (-OH) groups on the sugar moiety into aldehyde groups (-CHO) but the substitution to carboxyl groups (-COOH) never happens [10]. In contrast, chromic acid finally oxidizes hydroxyl groups to carboxyl groups.

Utilization of periodic acid instead of chromic acid in Grocott stain for fungi has been reported so far, but the researchers commented that the results were not fully applicable [2, 24]. In the present study, however, the combination of preheating and oxidation in 1% periodic acid solution for 10 min yielded satisfactory results for the consistent and stable visualization of a variety of fungi in pathological lesions. Carson *et al.* [1] reported the use of warm periodic acid as an oxidant, but satisfactory results were hardly obtained in our trial. Heat pretreatment is commonly used for epitope retrieval in immunostaining [20]. The heating process allegedly loosens and even removes formalin-induced methylene-bridges among tissue proteins [3]. The heat-induced change in the spatial structure of fungal proteins may thus contribute to the vulnerability to periodate oxidation. It should be noted that the stainability of *Mucor* hyphae was greatly enhanced in our modification. With conventional Grocott stain, *Mucor* hyphae are often stained weakly, probably due to the thinness of the cell wall [7, 23]. Kawabata *et al.* [12] briefly described in Japanese the enhanced staining of *Mucor* hyphae by double treating sections with heating and periodic acid oxidation. They simply aimed at enhancing the staining intensity of *Mucor* hyphae, so that chromic acid oxidation was preserved for demonstrating *Candida* spp. and *Aspergillus* spp.

Regarding the heating method, the electric jar, microwave oven, and pressure pan all gave satisfactory results, and all 4 types of soaking solution (tap water, distilled water, 10 mM citrate buffer, pH 6, and 1 mM EDTA, pH 8) provided comparable effects. Needless to say, tap water should be chosen as a soaking solution. The heating method can be selected, depending upon which one the laboratory technicians is most accustomed to use. We recommend pressure pan heating in tap water, because of its simplicity and convenience once one gets used to it.

During trial and error, we first succeeded in reducing the concentration of chromic acid from 5% to 1%. We discovered that warming at 60°C was needed, and we confirmed the repeated use (reuse and/or recycle) of the 1% chromic acid solution for a long period of time. Because of the high melting temperature of chromic acid at 196°C [22], warming at 60°C does not accelerate the risk of vaporization of the dangerous metal into air. It should be noted that the reduction in chromic acid from 5% to 1% had little effect on the perceived toxicity of the chemical. Referring to the Exposure Standards from the Hazardous Substances Information System (HSIS) [18], such risk phases still exist: R23 (toxic by inhalation), R36/37/38 (irritating to eyes, respiratory system and skin), R42/43 (may cause sensitization by inhalation and skin contact), R45 (may cause cancer), and R46 (may cause heritable genetic damage).

We also referred to the report by Hayashi *et al.* [8], who improved PAM stain by enhancing the silver reaction through the pretreatment with thiosemicarbazide after periodic acid oxidization. However, thiosemicarbazide belongs to the hazardous chemical category and so we abandoned using it.

Our new "ecological" Grocott stain is definitely friendly to both environment and human body. Total staining time is on a par with conventional Grocott stain. Since our modified technique requires no special equipment or additional reagents, we believe our simple and practical method is easily applicable worldwide.

V. Competing Interest Statement

The authors have no conflict of interests.

VI. Acknowledgments

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VII. References

1. Carson, F. L., Fredenburgh, J. and Maxwell, J. E. (1999) Inconsistent detection of *Histoplasma capsulatum* with periodic acid oxidation in the Grocott methenamine-silver nitrate (GMS) fungus stain. *J. Histotechnol.* 22; 119–122.
2. Carson, F. L. and Richmond, R. S. (2010) Periodic acid cannot replace chromic acid in Grocott's method for fungi. *Biotech. Histochem.* 85; 270.
3. Cattoretti, G. and Suurmeijer, A. J. H. (1995) Antigen unmasking on formalin-fixed paraffin-embedded tissues using microwaves: a review. *Adv. Anat. Pathol.* 2; 2–9.
4. Gomori, G. (1946) A new histochemical test for glycogen and mucin. *Am. J. Pathol.* 10; 177–179.
5. Gridley, M. F. (1953) A stain for fungi in tissue sections. *Am. J. Pathol.* 23; 303–307.
6. Grocott, R. G. (1955) A stain for fungi in tissue sections and smears using Gomori's methenamine-silver nitrate technic. *Am. J. Pathol.* 25; 975–979.
7. Guarner, J. and Brandt, M. E. (2011) Histopathologic diagnosis of fungal infections in the 21st century. *Clin. Microbiol. Rev.* 24; 247–280.
8. Hayashi, I., Tome, Y. and Shimosato, Y. (1989) Thiosemicarbazide used after periodic acid makes methenamine-silver staining of renal glomerular basement membranes faster and

cleaner. *Stain Technol.* 64; 185–190.
9. Isobe, Y., Chen, S. T., Nakane, P. K. and Brown, W. R. (1977) Studies on translocation of immunoglobulins across intestinal epithelium. I. Improvements in the peroxidase-labeled antibody method for application to study of human intestinal mucosa. *Acta Histochem. Cytochem.* 10; 161–171.
10. Jackson, E. L. (2011) Periodic acid oxidation. Wiley Online Library 15 MAR 2011, DOI: 10.1002/0471264180.or002.08.
11. Jones, D. B. (1953) Glomerulonephritis. *Am. J. Pathol.* 29; 33–51.
12. Kawabata, Y., Igarashi, H. and Sugimura, H. (2010) Pretreatment of Grocott stain for identifying *Zygomycetes* (*Mucor*). *Med. Technol.* 38; 1166–1168 (in Japanese).
13. Kligman, A. M., Meson, H. and DeLamater, E. D. (1951) The Hotchkiss-McManus stain for the histopathologic diagnosis of fungus diseases. *Am. J. Clin. Pathol.* 21; 86–91.
14. Mahan, C. T. and Sale, G. E. (1978) Rapid methenamine silver stain for *Pneumocystis* and fungi. *Arch. Pathol. Lab. Med.* 102; 351–352.
15. Musto, L., Flanigan, M. and Elbadawi, A. (1982) Ten-minute silver stain for *Pneumocystis carinii* and fungi in tissue sections. *Arch. Pathol. Lab. Med.* 106; 292–294.
16. Pintozzi, R. L. (1978) Modified Grocott's methenamine-silver nitrate method for quick staining of *Pneumocystis carinii*. *J. Clin. Pathol.* 31; 803–805.
17. Sadhakar, P., Ramadurai, L. and Anuradha, C. S. (2006) Allied's Environmental Education. Allied Publishers, New Dehli, India.
18. Safe Work Australia (2014) http://hsis.safeworkaustralia.gov.au/
19. Senba, M. (1983) A reliable silver staining method for Pneumocystis carinii and reticulin fibers in histologic sections. *Acta Histochem. Cytochem.* 16; 169–171.
20. Shi, S. R., Cote, R. J. and Taylor, C. R. (1997) Antigen retrieval immunohistochemistry: past, present, and future. *J. Histochem. Cytochem.* 45; 327–343.
21. Smith, J. M. and Hughes, W. T. (1972) A rapid staining technique for *Pneumocystis carinii*. *J. Clin. Pathol.* 25; 269–271.
22. Toxnet (Toxicology Data Network) (2009) Chromic acid. http://toxnet.nlm.nih.gov/cgi-bin/sis/search/a?dbs+hsdb:@term+@DOCNO+6769
23. Tsutsumi, Y. (2004) Pathology of Infectious Diseases. http://info.fujita-hu.ac.jp/~tsutsumi/
24. Youngberg, G. A., Wallen, E. D. B. and Giorgadze, T. A. (2003) Narrow-spectrum histochemical staining of fungi. *Arch. Pathol. Lab. Med.* 127; 1529–1530.

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