〈Brief Note〉

Effectiveness of BD CytoRich™ blue method as a quick Kohn's one-step staining method for *Entamoeba histolytica* trophozoites and *Giardia intestinalis* cysts

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Summary Heidenhain Iron-hematoxylin Staining (HIH) has been used to prepare permanent stained specimens of intestinal protozoa. But, HIH cannot be used at present due to environmental considerations because it is used mercuric chloride in this dyeing process. For this reason, Kohn's one-step stain has been used instead of HIH. However, this staining takes several hours to prepare the specimen. In this study, we devised a method to rapidly prepare a specimen using pretreating the sample by BD CytoRich™ Blue method. By using this method, it was possible to reduce the staining time to 15 minutes for trophozoites and 30 minutes for cysts, respectively (Normal staining times are 2-4 hours). And the sample preparation time was about 90 minutes was drastically reduced.

Key words: BD CytoRich™, *Entamoeba histolytica*, *Giardia intestinalis*, Intestinalis protozoa, Kohn's one-step staining

1. Introduction

Traditionally, Heidenhain Iron-hematoxylin Staining (HIH) has been the preferred method for preparing permanent specimens of intestinal protozoa. However, since mercuric chloride is used in the pretreatment process of this dyeing, it is not used at present because of consideration for the environment[1]. Kohn's one-step staining[2] is now used in its place, but both methods require a long staining period.

In recent years, BD CytoRich™ Blue (Becton, Dickinson and Company, BD Life Sciences-Integrated Diagnostic Solutions, Durham, NC) (CRB) method has expanded outside of the gynecology field, and it is now being used for pathological tissue preparation and a wide variety of other applications, with favorable results reported[3-5]. However, this paper is the first report using this

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method in the parasitology field, especially for intestinal protozoa.

In this study, a CRB method was used for Kohn's one-step staining of *Entamoeba histolytica* trophozoite specimens stored in a proper sodium acetate - acetic acid - formalin (SAF:1.5g sodium acetate, 2.0mL acetic acid and 4.0mL formalin in 92.0mL distilled water) in trophozoites and *Giardia intestinalis* cysts in a 10% formalin solution, after pre-processing was completed. This staining method was introduced recently as a replacement for HIH staining.

Kohn's one-step staining normally requires 2 - 4 hours of staining time, but for this study, when the trophozoites specimens were stained for 15 minutes and the cysts were stained for 30 minutes, favorable permanent specimens were created, and the specimen creation process was complete in around 90 minutes.

2. Materials and Methods

Entamoeba histolytica trophozoites (subculture using *Escherichia coli* and rice flour) fixed with a SAF solution and *Giardia intestinalis* cysts fixed with 10% Formalin solution were the materials used. The procedure of CRB method is briefly described below. This is a simple for the this method. For more details, see the manufacture's protocol. The minimum required for using this method is a slide rack, settling chamber, BD SurePath™ (Becton, Dickinson and Company, BD Life Sciences-Integrated Diagnostic Solutions) precoat slide glass and CRB solution.

(1) Centrifuge (2000 rpm, 10 minutes) the SAF solution and the storage solution fixed with 10% Formalin, collect supernatants, and set the total volume to 1 mL.
(2) Mix in an equal amount of the CRB solution.
(3) Centrifuge and collect the sediment.
(4) Add 5 mL of CRB preservative to the sediment and let sit for at least 30 minutes.
(5) Centrifuge and collect the sediment.
(6) Add 6 mL of purified water to the sediment, centrifuge (2,000 rpm, 5 minutes), then collect the sediment.
(7) Add 300 μL of purified water to the sediment, then mix well.
(8) Collect 300 μL with a micro-pipette add to the BD settling chamber and let sit for 10 minutes.
(9) Turn the slide rack upside down and discard excess water.
(10) Wash with 95 - 100% ethanol.
(11) Turn the slide rack upside down and discard ethanol, then proceed to Kohn's one-step staining series.

The Kohn's Stain Solution (Muto Pure Chemicals, LTD., Tokyo, Japan) used for this study and was filtered before use. The staining method was modified using Kohn's one-step staining method as a reference, slight changes were made and the dehydration time was extended. (Fig. 1) In addition, the ideal staining time for Chlorazol Black E (Kohn's stain solution) was determined from the following options: 2 hours, 1 hour, 45 minutes, 30 minutes, 15 minutes, 10 minutes, and 5 minutes.

In addition, the sample fixation state of the BD SurePath™ precoat slide used with CRB was compared to other slide glass types fixation states.

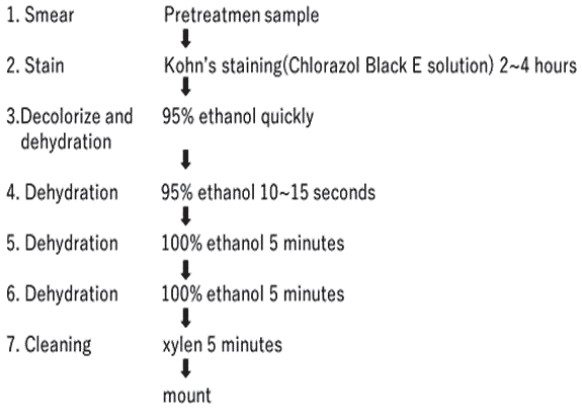

Fig. 1 Modified Kohn's one-step staining procedure. One more dehydration process is added to the standard method.

3. Results

For the *Entamoeba histolytica* trophozoites specimens, within the time intervals ranging from 30 minutes to 2 hours, hyper staining was observed, and

the internal elements of cells such as nucleus could not be confirmed. In addition, for staining intervals ranging from 5 to 10 minutes staining was pale and the internal structure of the trophozoite specimens was difficult to confirm. However, with 15 minutes of staining, the nucleus was clearly stained and the karyosome of *Entamoeba histolytica* could be observed at the center of nucleus (Fig. 2).

Although *Giardia intestinalis* cysts were stained in the same way as *Entamoeba histolytica*, structures such as the nucleus, karyosome and curved bristle could all be confirmed on the 30-minute staining interval specimen (Fig. 3).

Entamoeba histolytica trophozoite specimens were used to confirm the fixation state of the BD SurePath™ pre-coated slide glass samples. For the same sample volume of 15-minute staining specimens, sample fixation status for SurePath™ was compared with New Silane II coated slide glass and water excising slide glass.

When BD SurePath™ precoated slid glass was compared with the other slide glass types, the sample fixation was drastically superior (Fig. 4).

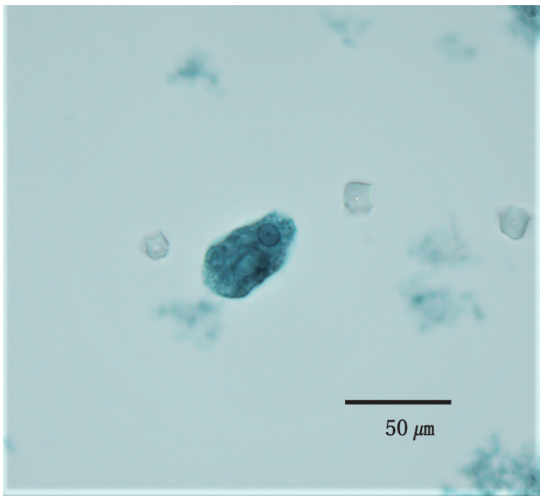

Fig. 2 Trophozoite of *Entamoeba histolytica* stained with Kohn's one-step staining for 15 minutes, showing nucleus and karyosome distinctly.

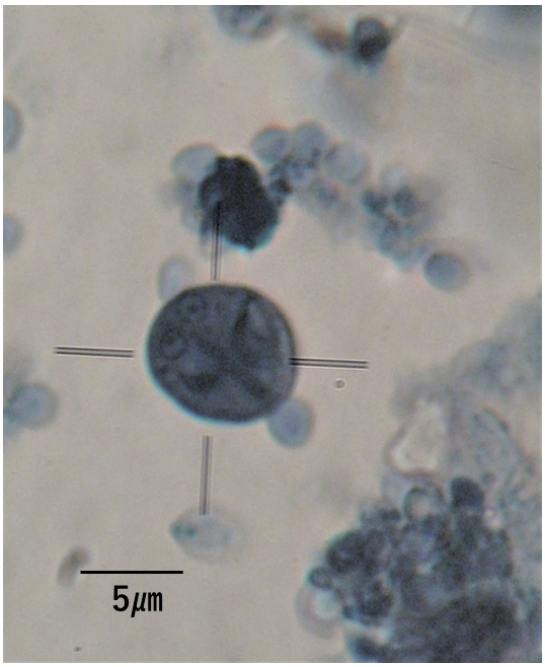

Fig. 3 *Giardia intestinalis* cyst stained with Kohn's one-step staining for 30 minutes, showing nucleus, karyosome, part of flagella and curved bristle distinctly.

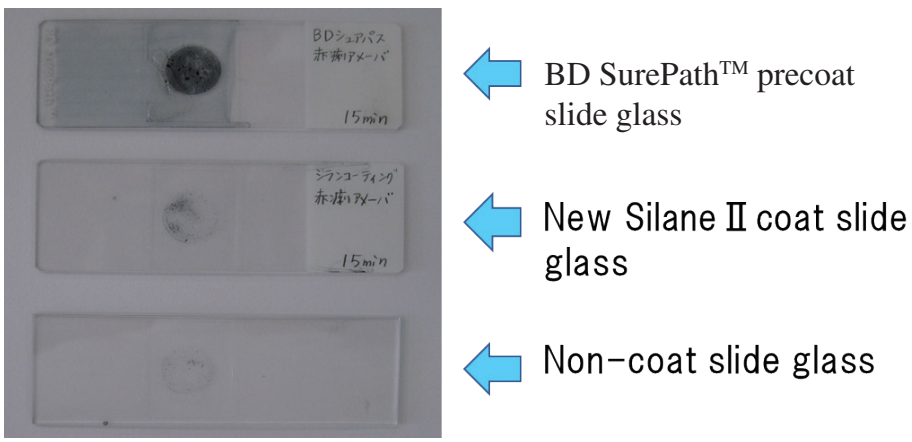

Fig. 4 Comparison of three slide glasses for sample fixation. The sample fixation of BD SurePath™ precoat slide glass is drastically superior to other slide glass types.

4. Discussion

When staining trophozoite of *Entamoeba histolytica* and *Giardia intestinalis* cyst, wet fixation, in which a stool sample is applied directly to the slide glass, is the fundamental method[6]. Although inspection immediately after the stool sample is received is ideal when using this method, Formalin fixation and SAF fixation can be used to preserve the protozoa, as seen in this study. With this technique, permanent stained specimens can be created later during unoccupied work hours.

Since using the CRB method enable even, single-layer application of samples to the slide glass, protozoa detection is easy. In addition, due to the drastic decrease in specimen preparation time, the same number of permanent specimens can be created more easily than with traditional methods, which is a significant advantage when using these specimens as a means for definitive diagnosis.

Although this report used different fixation methods for trophozoite of *Entamoeba histolytica* and *Giardia intestinalis* cyst, this is because the Formalin fixation samples for trophozoites of *Entamoeba histolytica* were poor. In addition, because acquiring fresh *Giardia intestinalis* cyst samples was difficult, SAF fixation samples could not be prepared, and this was also a factor. As soon as samples are acquired, further considerations with SAF fixation solution will be added.

5. Conclusion

This study showed that by using the CRB method for pre-processing and carrying out Kohn's one-step staining, the time required for staining and specimen preparation can be drastically reduced while achieving the same kind of staining images as standard Kohn's one-step staining methods.

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Conflicts of interest

The author has no conflicts of interest.

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