Research Article

Brief Report: Evaluation of Ziehl-Neelsen Slide Re-staining with 0.4% Malachite Green for the Identification of *Cyclospora cayetanensis* Oocysts

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Abstract

Cyclospora cayetanensis oocysts are commonly identified using Ziehl-Neelsen acid-fast staining. However, slides stored for prolonged periods often exhibit color fading, reducing visual clarity and limiting their suitability for training and educational purposes. This study evaluated the effectiveness of re-staining with 0.4% malachite green to enhance contrast and visualization of oocysts on faded slides. A quantitative experimental laboratory design was employed. Ten positive slides stored at the Clinical Parasitology Laboratory, Faculty of Medicine, Universitas Indonesia, were selected based on the degree of fading and staining quality. Slides were cleaned with methanol to remove residual immersion oil prior to re-staining with 0.4% malachite green for 30 seconds. Following drying, slides were examined under a light microscope, and oocysts were manually counted. Re-staining restored the background to a bluish-green hue, improving visual contrast and facilitating oocyst identification. Re-staining Ziehl-Neelsen slides with 0.4% malachite green is a simple and effective method for improving the visualization and identification of C. cayetanensis oocysts.

Keywords: *Cyclospora cayetanensis, Ziehl-Neelsen, malachite green, re-staining, oocyst enumeration.*

Laporan Singkat: Evaluasi Pewarnaan Ulang Sediaan Ziehl-Neelsen dengan Malachite Green 0,4% untuk Identifikasi Ookista *Cyclospora cayetanensis*

Abstrak

Ookista Cyclospora cayetanensis dapat diidentifikasi menggunakan pewarnaan tahan asam Ziehl-Neelsen. Namun, preparat yang disimpan dalam waktu lama sering mengalami pemudaran warna, sehingga menurunkan kejelasan visual dan membatasi penggunaannya untuk keperluan pendidikan dan pelatihan. Penelitian ini bertujuan mengevaluasi efektivitas pewarnaan ulang menggunakan malachite green 0,4% untuk meningkatkan kontras dan visualisasi ookista pada preparat yang telah memudar. Penelitian menggunakan desain laboratorik eksperimental kuantitatif. Sepuluh preparat positif yang tersimpan di Laboratorium Parasitologi Klinik, Fakultas Kedokteran Universitas Indonesia, dipilih berdasarkan tingkat pemudaran dan kualitas pewarnaan. Preparat terlebih dahulu dibersihkan dengan metanol untuk menghilangkan sisa minyak imersi, kemudian diwarnai ulang menggunakan malachite green 0,4% selama 30 detik. Setelah dikeringkan, preparat diamati menggunakan mikroskop cahaya dan jumlah ookista dihitung secara manual. Pewarnaan ulang mengembalikan warna latar menjadi hijau kebiruan, sehingga meningkatkan kontras visual dan mempermudah identifikasi ookista. Pewarnaan ulang preparat Ziehl-Neelsen dengan malachite green 0,4% merupakan metode yang sederhana dan efektif untuk meningkatkan visualisasi dan identifikasi ookista C. cayetanensis.

Kata kunci: *Cyclospora cayetanensis, Ziehl-Neelsen, malachite green, pewarnaan ulang, enumerasi ookista.*

Introduction

Cyclospora cayetanensis is a coccidian parasite that causes cyclosporiasis, a diarrheal illness transmitted through contaminated food or water. While infections in immunocompetent individuals may be asymptomatic, vulnerable populations such as children, the elderly, and immunocompromised individuals often experience prolonged watery diarrhea, abdominal cramps, weight loss, and low-grade fever. Because outbreaks are frequently linked to fresh produce, timely and accurate diagnosis is essential for effective treatment and for guiding public health interventions.[1-3]

Laboratory detection of *C. cayetanensis* remains challenging due to the small size of its oocysts (8–10 µm) and their inconsistent staining characteristics, which often result in underdiagnosis during routine ova and parasite examinations. Conventional microscopic methods, including modified acid-fast staining such as Ziehl-Neelsen (ZN), are widely employed, though oocysts may appear pink to red or remain unstained, against a blue or green background depending on whether methylene blue or malachite green is used as the counterstain. Autofluorescence under ultraviolet light provides an additional diagnostic feature, but confirmation of morphology by light microscopy is required to avoid misidentification. Molecular techniques such as polymerase chain reaction (PCR) offer high sensitivity and specificity, yet their availability is often limited to reference laboratories.[4-7]

Despite these diagnostic approaches, stained fecal smears used for microscopic identification may degrade over time, particularly through fading of background coloration, which compromises slide quality for teaching, archiving, and laboratory quality assurance. This deterioration is especially problematic in regions where cyclosporiasis cases are rare, as archived slides serve as critical references for training and diagnostic validation.[8,9] Addressing this gap, the present study evaluates the use of 0.4% malachite green as a re-staining agent to restore faded ZN acid-fast slides and improve visualization of *C. cayetanensis* oocysts.

Methods

A quantitative experimental laboratory study was conducted utilizing archived *C. cayetanensis*-positive slides obtained from the Parasitology Laboratory, Faculty of Medicine, Universitas Indonesia. Samples were collected between 2015 and 2020. Inclusion criteria comprised slides exhibiting a faded pink-red background and containing two complete smears per patient, while slides with green or blue background coloration were excluded.

All slides were initially examined under light microscopy at 400× magnification. Oocyst appearance and background staining were evaluated to assess slide quality. Slides were classified as good when oocysts were clearly visible, with well-defined morphology and pink-to-red staining against a green background produced by malachite green counterstaining. Slides were classified as poor when the background appeared faded and red or magenta in color, reducing contrast and making oocyst identification more difficult.

Poor-quality slides were selected for re-staining. Residual immersion oil was removed by cleaning the slides with methanol, followed by re-staining with 0.4% malachite green for 30 seconds. The slides were then rinsed with running tap water and air-dried at room temperature. Following re-staining, the slides were re-examined under light microscopy at 100× and 400× magnifications.

Oocyst density was determined by counting *C. cayetanensis* oocysts across twenty fields of view at 400× magnification, starting from a designated point. Enumeration was performed manually using a tally counter. The data were tabulated and analyzed using Microsoft Excel.

Results

Of the 78 archived *Cyclospora cayetanensis*-positive fecal smear slides, 10 (12.5%) met the inclusion criteria, characterized by faded pink-red backgrounds. Three slides were prepared in 2019, and another three were prepared in 2015. Re-staining with 0.4% malachite green successfully restored the

background to a bluish-green hue, thereby enhancing contrast with the pink-red oocysts and markedly improving visual identification and manual enumeration under light microscopy (Figure 1).

In Figure 1A, a faded acid-fast stained slide displays two round oocysts with double walls and diameters of 8–10 µm. The upper oocyst contains internal granules and appears pink (solid arrow), while the lower oocyst is empty and unstained (dashed arrow). The image was captured at 400× magnification with a scale bar of 1:2. In Figure 1B, the same slide following re-staining demonstrates a bluish-green background that provides sharper contrast, allowing clear visualization of a pink-red *C. cayetanensis* oocyst (green arrow).

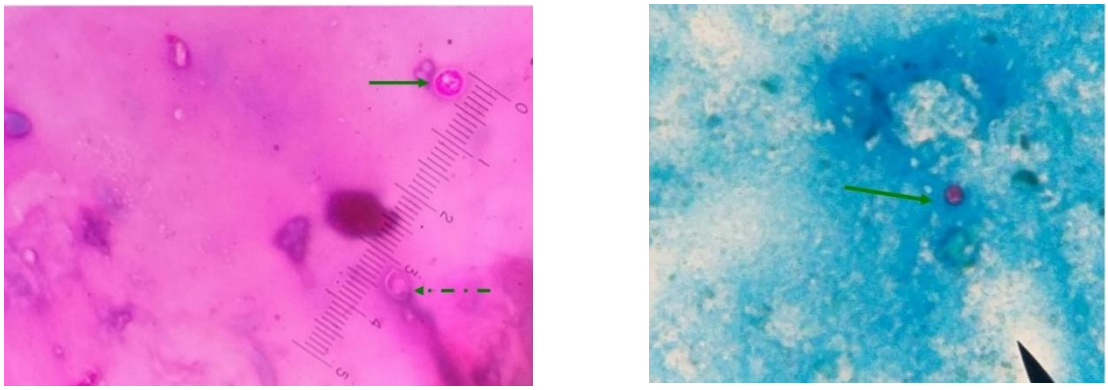

Figure 1. *C. cayetanensis* Oocysts Before (A) and After Re-staining with 0.4% Malachite Green (B)

The number of *C. cayetanensis* oocysts observed per slide ranged from one to 55, as detailed in Table 1. This finding suggests that re-staining improved visualization while maintaining oocyst detectability.

Table 1. Characteristics of Re-stained Slides and Oocyst Density

Slide Code	Storage Duration (months)	Oocyst Density
1	36	2
2	48	6
3	5	55
4	6	2
5	26	1
6	8	3
7	47	3
8	54	1
9	48	2
10	11	1

Discussion

Cyclosporiasis detection methods have been developed based on the identification of oocyst morphology through direct and/or staining microscopic examination, as well as molecular testing.[3] *C. cayetanensis* oocysts in clinical samples, such as human stool, are identified based on their size and other morphological characteristics.[10] The oocyst walls of *C. cayetanensis* contain acid-fast lipids that facilitate detection by acid-fast staining, particularly with modified acid-fast stains. Unlike the ZN method used for mycobacteria, the modified ZN stain does not require heating the reagents.[6]

The steps for acid-fast staining involve flooding a smear with strong carbol fuchsin, decolorizing with 5% sulfuric acid in alcohol, and counterstaining with methylene blue or malachite green, producing a blue or green background. Carbol fuchsin penetrates the lipoidal wall and enters the cytoplasm, causing all cells to appear red. Another type of modified acid-fast stain for detecting parasites uses decolorization with 1–3% H_2SO_4, avoiding the use of alcohol. *C. cayetanensis* oocysts are resistant due to the large amount of lipoidal material in their cell walls, which prevents penetration of the decolorizing solution.[11,12]

Most re-stained slide samples were aged >40 months, suggesting that factors such as storage conditions may have a deteriorating effect on stains. According to Ratsavong et al,[13] high environmental temperatures (35–40°C) and high humidity, common in the Indonesian climate, have

a detrimental effect on staining, with colors lasting only 2–4 weeks. Other factors, such as immersion oil remaining on slides, may negatively affect acid-fast staining and overall quality. Acid-fast stained slides should therefore be stored in a humidity-controlled area, even if not air-conditioned, to prolong stain lifespan.[14]

Before re-staining, slides should be cleaned with xylene or xylene-based strips to remove excess immersion oil. In this study, methanol was used as a cleaning agent to remove residual immersion oil prior to re-staining**.** A microwave method may also be employed to clean samples for re-staining; this method has been proven safe in laboratory environments. However, it may result in crystallization of the stain on the film due to evaporation and drying.[15]

No evidence was found regarding the effect of counterstains—such as using methylene blue instead of malachite green—or the amount applied on staining and re-staining quality. Only decolorized cells absorb the counterstain, appearing blue, while acid-fast cells retain the red color.

Although staining generally produced good results, complications were noted in staining methods and microscopic observation. One complication of re-staining was that malachite green applied to deteriorated samples could overlap with the former stain, making oocyst identification difficult.[15] Some samples also contained transparent oocysts (“ghost cells”) due to variable dye uptake, or appeared as non-refractile glassy spheres, which further complicated identification.

Other factors, such as experience and training, influence the quality and sensitivity of microscopic enumeration. Because microscopy is highly subjective, more experienced and better-trained specialists yield more reliable results.[14] Stool smears for staining can be prepared directly from fresh stool or from concentrated stool obtained through water-ether sedimentation, formalin-ether sedimentation, or sucrose centrifugal flotation techniques, which enhance sensitivity for detecting small numbers of *C. cayetanensis* oocysts.[17]

However, no data were available regarding whether stool smears originated from fresh or concentrated samples. Since the number of oocysts shed in stool may be relatively low, examination of multiple stool samples is often required to diagnose cyclosporiasis.[18]

This study suggests that re-staining faded acid-fast stained *C. cayetanensis* slides with 0.4% malachite green was successful and improved the visualization of oocysts and facilitated their enumeration. Future refinements may involve controlled decolorization prior to re-staining to reduce overlapping, though this carries the risk of removing the red color from oocysts. Incorporating multiple observers in microscopic visualization may also reduce subjectivity and error. Ultimately, while staining remains a valuable tool, integration with molecular methods could provide more robust diagnostic confirmation, particularly in low-resource settings where microscopy is the primary method of detection. The application of a mounting medium may help preserve stain color and oocyst morphology in permanent slides intended for long-term archiving and teaching purposes.

Several limitations should be acknowledged. First, the study did not document whether stool smears were prepared directly from fresh stool or from concentrated samples, which may affect sensitivity and comparability of results. Second, the majority of re-stained slides aged above 40 months, raising concerns about the influence of long-term storage and environmental conditions on stain deterioration. Third, the evaluation relied heavily on microscopic observation, which is inherently subjective and dependent on the experience of the examiner; inter-observer variability was not systematically assessed. Fourth, the study did not explore alternative counterstains or systematically compare malachite green with other staining agents, limiting generalizability of the re-staining approach. Finally, molecular confirmation of oocyst identity was not performed, which may restrict the diagnostic accuracy of the findings and their applicability to broader epidemiological contexts.

Conclusion

Faded *C. cayetanensis* slides originally stained with Ziehl-Neelsen acid-fast methods can be successfully re-stained using 0.4% malachite

green for 30 seconds. The background color was restored to a bluish-green hue, enhancing contrast with pink-red oocysts and improving visualization for diagnostic and archival purposes.

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Authors' Contributions

All authors contributed equally to the conception, design, data collection, analysis, interpretation, and manuscript preparation. Each author reviewed, approved, and accepted responsibility for the final manuscript.

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Data and Materials Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request. Archived slides are stored at the Parasitology Laboratory, Faculty of Medicine, Universitas Indonesia, but are not publicly accessible due to institutional restrictions.

Consent to Participate

Not applicable. This study did not involve human participants, as only anonymized archived laboratory materials were used.

Ethics Statement

This study did not require ethical approval because it utilized anonymized archived fecal smear slides collected previously for routine diagnostic purposes. No human or animal subjects were directly involved. The research was conducted in accordance with accepted international ethical guidelines.

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