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Research Article

Enhancing Detection of *Demodex Folliculorum* in Patients with Blepharitis Using Methylene Blue Dye

Hernandez-Francisco S[1], Gonzalez-Mondragon JE[1], Ramirez-Hernández R[1], Diener-Kudisch S[1,4], Mejia-Llanes DB[1], Nava-Castañeda A[1*]

[1]Oculoplastic Department, Instituto de Oftalmologia Fundacion Conde de Valenciana, Mexico City, Mexico

***Correspondence author:** Angel Nava Castañeda MD, PhD, Sc.D. and Oculoplastic Surgeon, Oculoplastic Department, Instituto de Oftalmologia Fundacion Conde de Valenciana, I.A.P. Chimalpopoca 14, Colonia Obrera, 06800. Mexico City, Mexico; Email: angellusnc@gmail.com

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Abstract

Introduction: *Demodex Folliculorum* is a prevalent ectoparasite of human pilosebaceous units and has been closely linked to chronic blepharitis. Conventional light microscopy is regarded as the gold standard for diagnosis; however, detecting the parasite can be challenging due to its translucent morphology, particularly for inexperienced observers. Purpose: This study aims to assess whether methylene blue staining enhances the microscopic detection of *Demodex Folliculorum* compared to conventional unstained examination in patients with blepharitis.

Methods: A prospective observational study was conducted involving patients exhibiting clinical signs of blepharitis. Four eyelashes per eyelid were epilated and examined under light microscopy before and after the application of 1% methylene blue. The total number of parasites and examination time were recorded. A paired Student's t-test was utilized to compare parasite counts before and after methylene blue staining. A p-value < 0.05 was considered statistically significant.

Results: A total of 167 participants with clinical signs of blepharitis were enrolled in the study, comprising 56 males (34%) and 111 females (66%), with a mean age of 60.75 ± 15.88 years. Among them, 141 individuals were found to have the Demodex parasite, including 49 males (35%) and 92 females (65%), with a mean age of 62.26 ± 14.13 years. The most reported clinical symptom was itching. Methylene blue staining significantly increased parasite detection in both eyelids compared to unstained microscopy (left eye: mean difference 1.96; right eye: 2.22; $p < 0.001$).

Conclusion: Methylene blue staining significantly enhances the microscopic detection of *Demodex Folliculorum*. Its low cost and ease of use render it a valuable adjunct for routine diagnostic evaluation.

Keywords: *Demodex Folliculorum*; Mites; Blepharitis; Methylene Blue; Microscopy; Eyelash Infestation

Introduction

Blepharitis is a chronic inflammatory condition affecting the eyelid margin and is among the most prevalent disorders encountered in ophthalmologic and dermatologic practice. Its etiology is multifactorial, encompassing bacterial colonization, meibomian gland dysfunction and parasitic infestation, particularly by *Demodex Folliculorum* [1,2]. *Demodex Folliculorum* is an obligate ectoparasite residing within human pilosebaceous units, predominantly in facial skin and eyelash follicles. While low-density colonization may be asymptomatic, increased mite density has been associated with inflammatory skin conditions such as rosacea, dermatitis and chronic blepharitis [3-5]. Several studies have demonstrated a higher prevalence and parasite load in patients with chronic or refractory blepharitis compared to healthy controls [6,7].

Microscopic examination of epilated eyelashes remains the gold standard for diagnosing ocular demodicosis [8]. However, detection can be challenging due to the translucent morphology of the parasite, overlapping debris and variability in observer experience, potentially leading to underdiagnosis or false-negative results [9]. To address these limitations, various adjunctive diagnostic techniques have been proposed, including *in-situ* visualization, modified epilation techniques and the use of staining agents to enhance contrast [10,11]. Methylene blue is a cationic dye widely used in microscopy as a contrast agent due to its affinity for nucleic acids and cellular structures.

Previous reports suggest that methylene blue staining may facilitate Demodex visualization by enhancing contrast without penetrating or altering the parasite's morphology [12,13]. The present study evaluates the diagnostic utility of methylene blue staining as an adjunct to conventional light microscopy for improving the detection of *Demodex Folliculorum* in patients with blepharitis.

Methodology

An observational and prospective study was undertaken, with the sample size determined based on incidence, necessitating a cohort of 200 participants. Inclusion criteria encompassed individuals over 18 years of age exhibiting collarettes, meibomian gland dysfunction, dry eye, telangiectasias on the eyelid margin or any other signs or symptoms indicative of blepharitis attributable to *Demodex Folliculorum*. Individuals with prior blepharitis treatment were excluded. A fixed number of four nonadjacent eyelashes per eyelid was selected as a standardized and clinically feasible sampling approach, consistent with previously described diagnostic methodologies [14,15]. This strategy allows reproducible comparison between samples while minimizing procedural variability. Additionally, as the primary objective of the study was to compare detection methods rather than quantify absolute parasite burden, a fixed sampling strategy was considered appropriate. These eyelashes were placed on glass slides without staining and examined via conventional light microscopy at magnifications of 40x and 100x. Parasite counts were recorded separately for the left and right eyelids.

Following the baseline unstained examination, a drop of 1% methylene blue solution was applied to the same eyelash samples. Microscopic evaluation was repeated under identical conditions and parasite counts were reassessed for each eyelid, facilitating paired comparison. All microscopic evaluations were conducted by two independent observers (ANC and JEGM) with expertise in ocular surface assessment. Observers were not masked to the staining condition, as the same eyelash samples were examined sequentially before and after methylene blue application. Final parasite counts were established by consensus. The primary outcome was the difference in *Demodex Folliculorum* mite counts before and after methylene blue staining for each eyelid. Secondary outcomes included the comparison of parasite burden between eyelids.

In the statistical analysis, quantitative variables were expressed as mean and Standard Deviation (SD). A paired Student's t-test was employed to compare parasite counts before and after methylene blue staining. A p-value < 0.05 was considered statistically significant. Statistical analysis was performed using Microsoft Excel (Microsoft, Redmond, WA, USA).

Ethical Statement

The study adhered to the tenets of the Declaration of Helsinki, approved by the Institutional Ethics Committee Board (Registration Number: CEI-2024/03/02). All participants provided written informed consent prior to enrollment.

Results

A total of 167 participants exhibiting clinical signs of blepharitis were enrolled in the study, comprising 56 males (34%) and 111 females (66%), with a mean age of 60.75 ± 15.88 years. 141 individuals were found to have the Demodex infestation, including 49 males (35%) and 92 females (65%), with a mean age of 62.26 ± 14.13 years. Conversely, 26 participants did not present with Demodex, of whom 7 were males (27%) and 19 females (73%), with a mean age of 52.81 ± 21.85 years. The most reported clinical symptoms among the blepharitis cohort included itching in 134 subjects, discharge in 75, eyelash loss in 28, tearing in 29, telangiectasia in 27 and a gritty sensation in 13 subjects. Within the Demodex-positive group, 112 reported itching, 65 discharge, 24 eyelash loss, 27 tearing, 24 telangiectasia and 12 a gritty sensation (Table 1). In the unstained condition, the mean parasite count was 4.25 ± 5.01 in the left eye and 4.85 ± 5.35 in the right eye. Following methylene blue staining, the mean parasite count increased to 6.21 ± 6.54 in the left eye and 7.07 ± 7.25 in the right eye. This represented a statistically significant increase in parasite detection for both eyes (left eye: mean difference 1.96, $p< 0.001$; right eye: mean difference 2.22, $p<0.001$) (Fig. 1). The increase in

parasite count corresponded to a moderate effect size, with a mean difference of 2.1 (95% CI: 1.4-2.8) for the left eye and 2.4 (95% CI: 1.6-3.2) for the right eye. Cohen's d values were 0.42 and 0.45, respectively, confirming the magnitude of the observed improvement. Methylene blue staining increased the proportion of positive infestation index cases from 79.4% to 88.7%, as shown in Table 2 and Fig. 2.

Variable	Blepharitis (n=167)	Demodex (n=141)	No Demodex (n=26)
Sex			
Female	111 (66%)	92 (65%)	19 (73%)
Male	56 (34%)	49 (35%)	7 (27%)
Age (Mean ± SD)	60.75 ± 15.88	62.26 ± 14.13	52.81 ± 21.85
Pathologies			
None	121 (72%)	-	-
Glaucoma	6 (3%)	6 (4%)	0 (0%)
Cataract	12 (7%)	10 (7%)	2 (8%)
Myopia	26 (15%)	21 (15%)	5 (19%)
Astigmatism	18 (10%)	13 (9%)	5 (19%)
Hyperopia	4 (2%)	4 (3%)	0 (0%)
Duration			
0 - 4 weeks	14 (8%)	13 (9%)	1 (4%)
> 4 weeks to 6 months	39 (23%)	33 (23%)	6 (23%)
> 6 months to 1 year	60 (35%)	51 (36%)	8 (31%)
> 1 year	54 (32%)	42 (30%)	10 (38%)
Symptoms			
Pruritus (Itching)	134 (80%)	112 (79%)	22 (85%)
Secretion/Discharge	75 (44%)	65 (46%)	10 (38%)
Loss of eyelashes	28 (16%)	24 (17%)	4 (15%)
Tearing	29 (17%)	27 (19%)	2 (8%)
Telangiectasia	27 (16%)	24 (17%)	2 (8%)
Grittiness / Foreign body sensation	13 (7%)	12 (9%)	1 (4%)

Table 1: Epidemiological and clinical data. Epidemiological and clinical characteristics of patients with blepharitis, stratified by the presence or absence of *Demodex Folliculorum*. Data are presented as number (percentage) or mean ± standard deviation. Variables include sex, age, associated ocular pathologies, duration of symptoms and clinical manifestations.

Method	Demodex n=141
Standard Method	
Infestation Index > 0.5 (Positive)	112
Infestation Index < 0.5 (Negative)	21
Methylene Blue Staining Method	
Infestation Index > 0.5 (Positive)	125
Infestation Index < 0.5 (Negative)	15

Table 2: Infestation index in both methods. The infestation index was defined as the ratio of the number of *Demodex Folliculorum* mites to the number of epilated eyelashes. An index > 0.5 was considered positive. Percentages are calculated based on the total number of Demodex-positive patients (n = 141). Methylene blue staining increased the proportion of positive cases compared to the standard method.

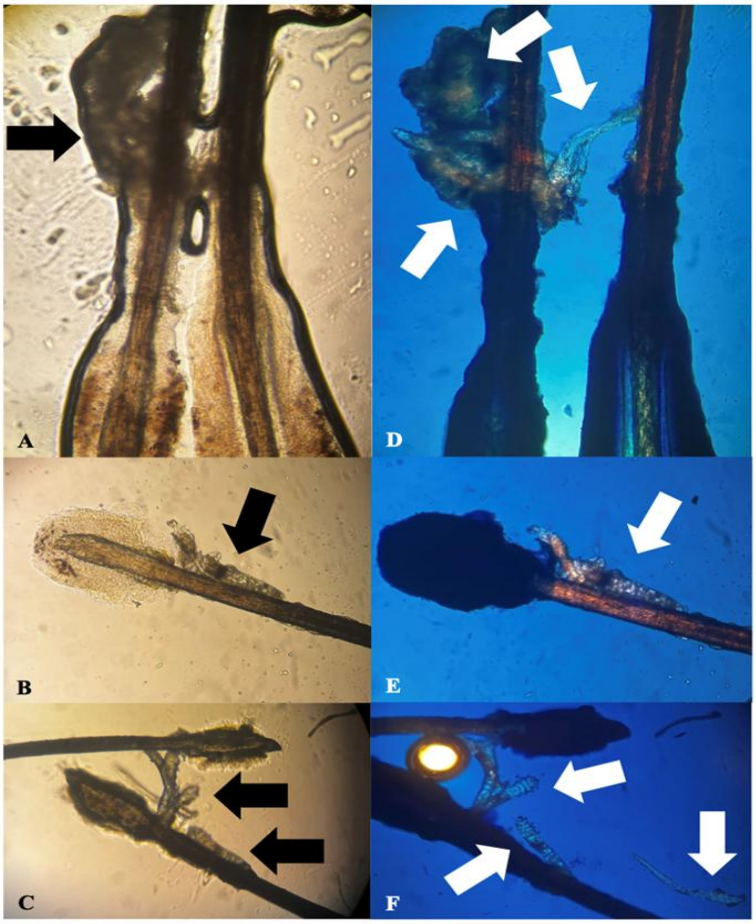

Figure 1: Evaluation and identification of Demodex on eyelashes. A, B and C using the traditional method. D, E and F through methylene blue staining.

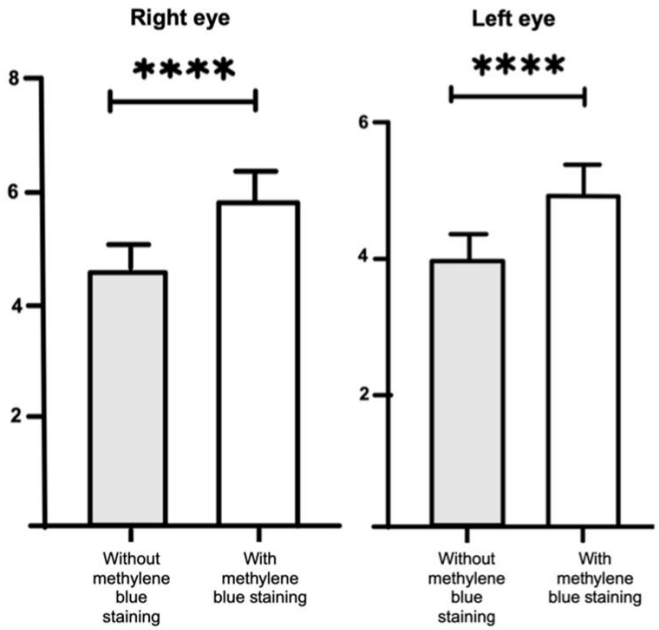

Figure 2: Mean *Demodex Folliculorum* counts per eyelid before and after methylene blue staining. Bars represent mean ± standard deviation. Methylene blue staining significantly increased parasite detection in both eyes. Statistical analysis was performed using a paired Student's t-test (****$p < 0.001$).

Discussion

This study demonstrates that methylene blue staining significantly enhances the microscopic detection of Demodex in patients with blepharitis compared to conventional unstained light microscopy. The increase in parasite counts likely improved visualization rather than true changes in infestation density, consistent with previous studies using contrast-enhancing techniques. Methylene blue improves delineation of mite structure without altering morphology, reducing false-negative results, particularly in cases with low parasite burden. This may facilitate more accurate diagnosis and support timely initiation of targeted treatments, such as lid hygiene protocols or acaricidal therapies. Improved detection may contribute to better disease classification and monitoring of treatment response, thereby optimizing patient management. An additional strength is the evaluation of both eyelids separately, revealing consistent improvement in parasite detection across eyelids. This supports the reproducibility of the technique and suggests that methylene blue staining can be reliably applied regardless of baseline parasite distribution [12]. Despite its strengths, this study has limitations. A potential limitation is the lack of masking during sample evaluation, as the same eyelash specimens were examined sequentially before and after staining. This may introduce observer bias and potentially overestimate parasite detection following methylene blue application. However, standardized evaluation criteria and the use of experienced observers may have mitigated this effect. Future studies incorporating masked evaluation are warranted. Additionally, this study was conducted at a single center with relatively homogeneous population, which may limit the generalizability of the findings to broader and more diverse populations. Therefore, caution should be exercised when extrapolating these results and multicenter studies are needs to further validate the external validity of these findings.

Although the number of epilated eyelashes varies across studies, the use of four nonadjacent lashes per eyelid represents a commonly accepted and standardized approach in clinical practice, supporting the methodological consistency and supports the reproducibility and internal validity of the present findings.

Conclusion

Methylene blue staining enhances the microscopic detection of *Demodex Folliculorum* by improving visualization of the semi-transparent parasite. This simple, cost-effective technique significantly increases detection rates and represents a practical adjunct to conventional light microscopy. Its use in routine clinical practice may improve diagnostic accuracy, reduce underdiagnosis and support timely and appropriate management of Demodex-associated blepharitis.

Conflict of Interest

The authors declare no conflict of interest. The authors are solely responsible for the content and writing of this manuscript.

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None.

Data Availability Statement

Not applicable.

Ethical Statement

The study adhered to the tenets of the Declaration of Helsinki, approved by the Institutional Ethics Committee Board (Registration Number: CEI-2024/03/02). All participants provided written informed consent prior to enrollment.

Informed Consent Statement

Informed consent was taken for this study.

Authors' Contributions

All authors contributed equally to this paper.

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