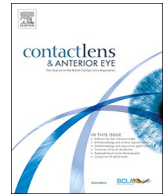




Contents lists available at ScienceDirect

Contact Lens and Anterior Eye

journal homepage: www.elsevier.com/locate/claeImproved *Demodex* diagnosis in the clinical setting using a novel *in situ* techniqueAlex Muntz^a, Christine Purslow^{b,c}, James S. Wolffsohn^d, Jennifer P. Craig^{a,*}^a Department of Ophthalmology, New Zealand National Eye Centre, The University of Auckland, New Zealand^b Thea, Ireland, UK^c School of Optometry and Vision Sciences, Cardiff University, UK^d Ophthalmic Research Group, Aston University, Birmingham, UK

ARTICLE INFO

Keywords:

Demodex
Dry eye disease
Eyelash
Blepharitis
Clinical diagnostic methods

ABSTRACT

Purpose: To compare existing and novel diagnostic techniques for confirming ocular *Demodex* infestation and to recommend the most reliable method for routine use by eye care practitioners, based on yield and clinical applicability.

Methods: Fifteen participants with a prior *Demodex* blepharitis diagnosis or featuring typical cylindrical dandruff (CD) collarettes, and seven healthy controls were enrolled. *Demodex* presence was assessed using five techniques, applied consecutively, on a minimum of two different eyelashes on each eyelid of every participant, for each test, *in situ*: 1. using fine-point forceps and 25–40x biomicroscopy magnification, by eyelash rotation as proposed by Mastrotta (ROT); 2. by removing cylindrical dandruff and exposing the eyelash insertion point at the lid margin (CDR); and 3. by laterally tensioning the eyelash (LET) following CDR. The typical appearance of cigar-shaped mite tails protruding from each assessed eyelash follicle was observed, and mite tails counted and averaged per participant for each assessment technique. 4. Lash epilation, and mite presence evaluated using bright-field microscopy at 10–40x magnification (EPI). 5. Finally, eyelash follicles were imaged using *in vivo* confocal microscopy (IVCM) and the images visually inspected for mite presence.

Results: In the *Demodex* group, the highest numbers of mites/eyelash were identified by LET (3.8 ± 1.4), versus CDR (2.4 ± 1.6) and ROT (1.1 ± 1.2), alone (all $p < 0.002$). An average of 1.0 ± 0.8 mites/lash was identified by EPI. IVCM failed to offer unequivocal evidence of *Demodex* presence even in confirmed cases.

Conclusions: A novel technique for the clinical diagnosis and grading of *Demodex in situ* is described. By removing cylindrical dandruff and applying static, lateral tension to the eyelash without epilation, large numbers of mites are visible at the exposed eyelash follicle. The proposed method is convenient and clinically applicable, requiring only forceps and 25–40x biomicroscope magnification, and allowing rapid, efficient evaluation of large numbers of eyelashes.

1. Introduction

Demodex mites commonly reside in the sebaceous glands of the scalp, face, ears, and in the meibomian glands and eyelash follicles [1]. Characteristic collarettes or cylindrical dandruff (CD) around the eyelash base are considered to be a sign of ocular demodicosis [2]. *Demodex* has a recognised association with anterior blepharitis and is believed to play a role in the perpetuation of ocular surface inflammation and dry eye disease [3–5]. Recently, numerous treatment [6,7] and diagnostic [3,8,9] options have emerged. While the topical application of tea tree oil (*Melaleuca alternifolia*) is the most commonly agreed management

strategy [7], a consensus on the optimal diagnostic technique for confirming mite presence, is lacking.

Traditionally, clinical diagnosis of *Demodex* infestation involves the epilation and microscopic evaluation of between 8 and 16 eyelashes per individual. Mites are visually identified by their characteristic morphology and motility [5]. Despite subsequent refinements to the sampling and counting methods in an attempt to increase yield [2,10–12], it is acknowledged that many mites can remain within the orifice following eyelash removal [5,13,14]. Mites embedded in the CD can be difficult to distinguish both *in vivo* and *ex vivo*, and addition of solvents to epilated lashes can cause mites to float away, decompose or perish,

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<https://doi.org/10.1016/j.clae.2019.11.009>

Received 19 June 2019; Received in revised form 11 October 2019; Accepted 18 November 2019

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resulting in an underestimation of mite count. In the clinical setting, repeated eyelash removal (e.g. for monitoring treatment efficacy) is uncomfortable and naturally undesirable to patients, and the time commitment and need for laboratory equipment (such as a 100-200x magnification light microscope, pipettes etc.) limits widespread integration of this technique into standard clinical care.

Proposed diagnostic alternatives for easier assessment and better patient comfort include a technique by which an eyelash is rotated around its own axis using forceps [13]. This motion reportedly “cores” *Demodex* from within the eyelash follicle, revealing mite tails at the insertion point of the lash. Using high biomicroscope magnification then allows the assessment of ocular *Demodex* infestation *in situ*. To date, adoption of the eyelash rotation technique in routine clinical applications appears not to be widespread, and no direct comparison between the various clinical diagnostic methods has been reported. Other diagnostic alternatives include *in vivo* confocal microscopy [3,8,9] although this requires costly equipment unavailable to the majority of clinicians.

This study compares established and adapted techniques with the aim of optimising *Demodex* diagnosis in the clinical setting.

2. Methods

2.1. Participants

This study followed the tenets of the Declaration of Helsinki and was approved by the University of Auckland Human Participants Ethics Committee (UAHPEC 013430). Informed consent was obtained from all participants prior to study enrolment.

Individuals of at least 18 years of age with a previous ocular demodicosis diagnosis by eyelash epilation, and/or signs of anterior blepharitis featuring typical cylindrical dandruff (CD) collarettes around the base of the eyelashes, were recruited. Additional age-matched participants free of anterior blepharitis were included as controls.

Participants presenting with major ocular or systemic disease, or reporting use of topical or systemic medications known to affect the eye, were excluded from the study.

2.2. Clinical measures

All participants completed the Ocular Surface Disease Index (OSDI) [15] and the 5-item Dry Eye Questionnaire (DEQ-5) [16].

A non-invasive tear film and ocular surface assessment was conducted using the Keratograph 5 M (Oculus, Wetzlar, Germany) and included measurement of the lower lid tear meniscus height, non-invasive tear break-up time (average of 3 measurements of first breakup detection), tear lipid layer quality [17] and bulbar and limbal hyperaemia.

Anterior segment biomicroscopy assessment was conducted and the following features graded on a scale from zero to three (0 = “none”; 1 = “mild”; 2 = “moderate”; 3 = “severe”, unless otherwise noted): cylindrical cuffing (typical collarette shape), collarette height (0 = < 1 mm, 1 = 1 mm, 2 = 2 mm, 3 = > 2 mm), collarette number (0 = 0, 1 = < 4, 2 = < 9, 3 = > 10), madarosis, trichiasis, lid margin erythema, thickening, surface telangiectasia, and irregularity. The number of meibomian glands yielding lipid secretion were assessed with the Meibomian Gland Evaluator (TearScience, Morrisville, NC) [18] and the expressed meibum quality graded.

Corneal staining was evaluated using sodium fluorescein (HUB Pharmaceuticals, Rancho Cucamonga, CA) with the aid of blue light and a yellow barrier filter, while conjunctival staining was assessed following application of lissamine green (HUB Pharmaceuticals, Rancho Cucamonga, CA) and white light. Both upper and lower lid margins were everted and lid wiper epitheliopathy graded according to the Korb scale [19]. The clinical application and evaluation of stains was performed in accordance with the procedures established in the TFOS DEWS II Diagnostic Methodology report [20].

2.3. *Demodex* assessment

Every participant underwent a 5-step eyelash assessment which was performed in the same test order each time, for least impact on the subsequent tests. Both upper and lower eyelids of both eyes of each participant were evaluated for *Demodex* presence using existing and refined clinical techniques for eyelash manipulation, epilation and *in vivo* confocal microscopy (IVCM).

The *in situ* techniques (1–3 below) were conducted under direct white light illumination and 25-40x biomicroscope magnification. Inspection of the upper lid eyelashes took place during participant downgaze, with the eyelids closed, while the lower eyelids were inspected during upgaze. A pair of sterilized, fine-tipped metal forceps (Altomed A5908 Jewellers Forceps No. 5 100 mm, Altomed, Bolton, UK) handled from the temporal side were used to manipulate the eyelashes during biomicroscopic observation. Mite tails protruding from the eyelash follicle resembling a typical cigar shape [13] were observed and counted for each procedure, ensuring only mite tails that could be confidently identified were counted. To enable secondary confirmation, procedures were digitally recorded as still images and/or videos with a biomicroscope-mounted camera (Topcon DC-4, Topcon, Japan or Canon T1i DSLR, Canon, Japan).

2.3.1. Rotation (ROT)

From each lid, two eyelashes featuring prominent cylindrical dandruff (CD) were identified and successively rotated for 30 s, as described by Mastrota [13]. During the rotation, the insertion point of the eyelash into the lid margin was continuously inspected for the characteristic cigar-shaped appearance of mite tails. Visible tails were counted and averaged across the 8 lashes to provide a mean count per lash.

2.3.2. Cylindrical dandruff removal (CDR)

Two different eyelashes with CD were selected. Using the tip of the forceps, the collarette base was secured and removed with a sliding motion along the eyelash. Where collarettes were fused with the adjacent cornified epidermis of the lid margin, removal typically resulted in (or required) separation of the fused tissue from the lid margin. By exposing the point of insertion of the eyelash into the lid margin in this way, protruding *Demodex* tails were counted (Video 1).

2.3.3. Lateral eyelash traction (LET)

The same two CD-free eyelashes were then subjected to a modified eyelash traction technique. In contrast to the technique described above (ROT), involving rotation of the lash around its own axis, the grasped eyelash this time was drawn only laterally, slowly alternating extension in nasal and temporal directions, under gentle constant tension, for 30 s. Mite tails emerging from the follicle during this lateral extension were counted (Fig. 1, Video 2).

2.3.4. Eyelash epilation and light microscopy (EPI)

Next, according to previously described methods [2,10], two different eyelashes per eyelid (eight eyelashes in total) were epilated and transferred to a glass slide. A standard laboratory bright-field microscope (Bresser, Rhede, Germany) at 100x, 200x and 400x magnification was used to count mites based on the characteristic morphological appearance and motility (Fig. 2). Average mite count per eyelash was reported.

2.3.5. *In vivo* confocal microscopy (IVCM)

Finally, *in vivo* confocal microscopy of the eyelash follicles was performed using the Heidelberg Retinal Tomograph III with a Rostock Corneal Module (Heidelberg Engineering, Dossenheim, Germany) to determine *Demodex* presence, as reported previously [8]. A single drop of 0.4 % oxybuprocaine (Benoxinate, Bausch & Lomb, Tampa, FL) anaesthetic was instilled bilaterally, to minimise the blinking reflex.

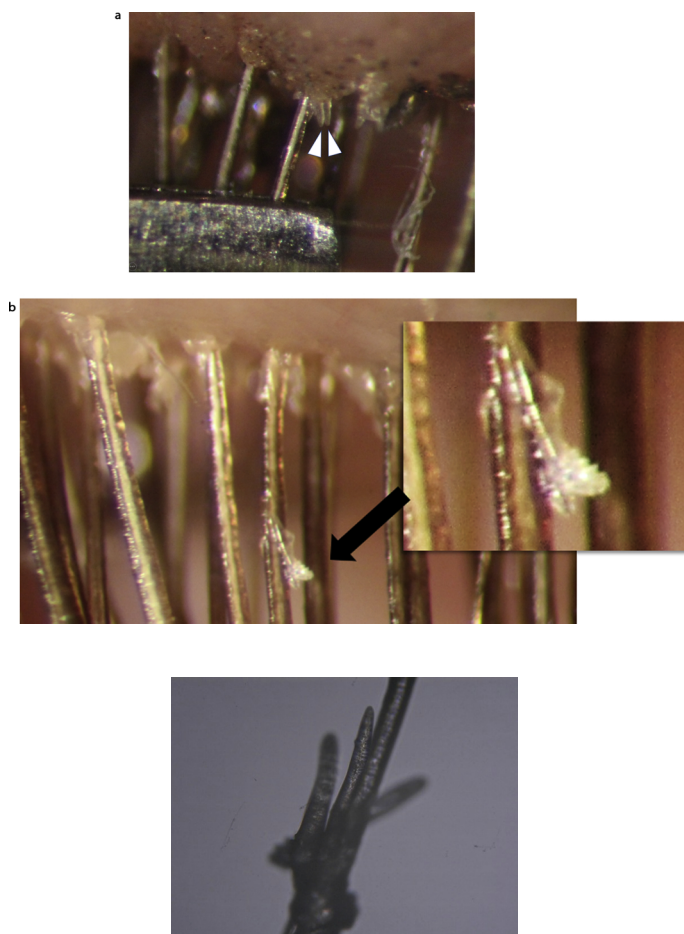


Fig. 2. Three *Demodex* mites observed on a single eyelash following epilation. Bright-field microscopy, 200x magnification.

Lubricating gel (Viscotears, Bausch & Lomb, Tampa, FL) was applied to a sterile TomoCap (Heidelberg Engineering, Dossenheim, Germany) and the lower and upper lid margins were inspected in turn. Images of the eyelash follicles were acquired and stored as digital image files and were visually assessed for mite presence.

Statistical analysis was performed with GraphPad Prism 7.02. Normally distributed continuous data underwent parametric statistical analysis and ordinal data were analysed with non-parametric tests. Normality was confirmed with the Kolmogorov-Smirnov test ($p > 0.05$). All tests were two-tailed and $p < 0.05$ was considered significant. Data are presented as mean \pm SD or median (IQR).

3. Results

The cohort of 22 participants was 59 % female and ranged in age from 33 to 80 years with a mean age of 59 ± 14 years. There were no significant differences in symptoms or tear film signs in those with and without signs of *Demodex* blepharitis (Table 1).

3.1. In situ *Demodex* presence

Epilation of 8 eyelashes per participant yielded a mean mite count of 1.0 ± 0.8 mites per eyelash in participants previously diagnosed with *Demodex* infestation (Table 1). No significant difference was noted between the epilation and rotation techniques ($p = 0.35$). Removing CD collarettes from the eyelash base (CDR) exposed almost twice as many mites than the rotation technique alone ($p = 0.0025$), while pulling the cleaned eyelash to the side (LET) revealed the highest numbers of *Demodex* tails ($p = 0.002$) (Fig. 3).

Fig. 1. a: two *Demodex* mite tails (white triangles) emerging from the eyelash orifice at the upper lid margin following cylindrical dandruff removal from the eyelash base. Using forceps to grasp the lash, lateral tension is applied to the eyelash. b: *Demodex* mite expelled onto eyelash after emerging from follicle during application of lateral tension. Biomicroscopy, 40x magnification. Inset: digitally magnified image.

Table 1

Study population demographics, tear film and ocular surface characteristics and *Demodex* mite count results. Data are presented as mean \pm SD, median (IQR), or as indicated in table. Asterisks denote statistically significant values ($p < 0.05$).

Characteristics and measures	Study group	Control group	Difference (p)
Sample size (n)	15	7	
Age (y)	57.6 ± 15.5	62.3 ± 11.4	0.437
Sex (% female)	53.3	71.4	0.647
OSDI score	21.0 ± 22.3	25.4 ± 12.5	0.217
DEQ-5 score	7.7 ± 5.5	10.7 ± 3.3	0.202
Tear film evaluation			
Tear meniscus height (mm)	0.3 ± 0.1	0.3 ± 0.1	0.454
NIK BUT (s)	10.6 ± 8.4	7.8 ± 6.0	0.477
Lipid layer grade (graded 0-5) [21]	3 (2-3)	2 (1-4)	0.371
Bulbar redness (graded 0-3)	1.2 ± 0.5	1.2 ± 0.4	0.854
Eyelash assessment (graded 0-3)			
Cylindrical dandruff	2 (1-3)	0 (0-0)	0.001*
Collarette height	1 (0-1)	0 (0-0)	0.033*
Collarette number	2 (1-3)	0 (0-1)	0.004*
Madarosis	1 (0-1)	0 (0-0)	0.038*
Trichiasis	1 (0-2)	0 (0-1)	0.126
<i>In situ</i> mite count (mites per lash)			
Rotation (ROT)	1.1 ± 1.2	0.1 ± 0.4	0.033*
Collarette removal (CDR)	2.4 ± 1.6	-	< 0.001*
Lateral eyelash traction (LET)	3.8 ± 1.4	0.4 ± 0.8	< 0.001*
<i>Ex vivo</i> mite count by eyelash epilation (EPI)	1.0 ± 0.8	0.1 ± 0.3	0.003*

3.2. In vivo confocal microscopy

Images acquired by IVCN were visually inspected for *Demodex* presence. Representative images, shown in Fig. 4, highlight the difficulty experienced in reliably confirming *Demodex* presence using this technique. An isolated eyelash, previously confirmed by bright-field microscopy to feature a viable *Demodex* mite, was embedded in lubricating gel and imaged directly on the TomoCap. Video 3 highlights the narrow depth of field of the IVCN relative to mite size and the poor distinction of the mite especially when aligned with the z-axis of the microscope.

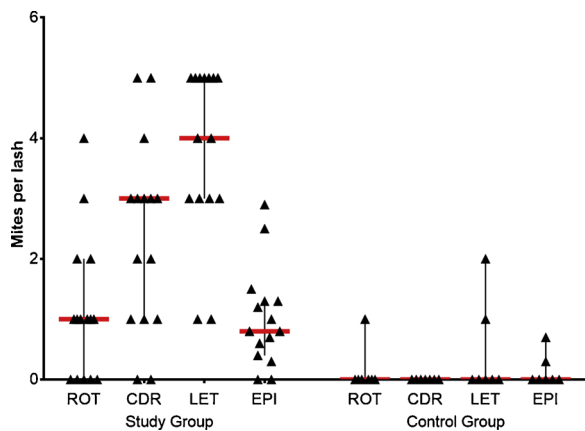


Fig. 3. Scatter plot for the average number of mites per lash recorded for each participant, by rotation, cylindrical dandruff-removal, lateral eyelash traction and epilation. The red horizontal line represents the median while the vertical line delimits the interquartile range.

4. Discussion

This study compared established and adapted techniques with the aim of optimising *Demodex* diagnosis in the clinical setting. The results indicate that higher numbers of *Demodex* mites than obtained with previously reported methods can be reliably obtained, *in situ*, by removing CD collarettes with fine-tipped forceps prior to applying lateral tension to the eyelash under high biomicroscopy magnification.

As recognised by Coston in his original monograph on *Demodex* and blepharitis, a significant disadvantage of the established eyelash epilation method appears to be that “only [mites] which happen to hold so tightly as to come out with the lash are seen; many more may be left in the follicle” [5]. This supposition likely led the author to propose the epilation of 16 lashes per patient to increase the chances of identifying

mites. Frequent mite miscounts and the inconvenience of (potentially repeated) removal of large numbers of lashes prompted Gao et al. and others to propose modifications to the original epilation and enumeration method [14]. One example is the rotation of the eyelash prior to epilation to encourage dislodging of the mites to increase mite yield on epilation. Using this technique, an average of 1.6 ± 2.9 mites per eyelash were reported by Gao et al. in a mixed sample of CD and CD-free patients. By adding sodium fluorescein to the sample, to increase the contrast between residual CD debris and mites during microscopy, Kheirkhah et al. reported a significant increase from 3.1 ± 2.5 to 4.4 ± 2.8 mites per lash in subjects with CD [10]. These values are somewhat higher than the average of 1.0 ± 0.8 mites per lash found in the present study using epilation, without the addition of sodium fluorescein.

It was possible, however, to significantly increase the yield with an adapted *in situ* method involving CD-removal followed by lateral eyelash traction. Not uncommonly five confirmed mites per lash could be distinguished in the *Demodex* group (in 0 % of cases using ROT, 13 % using CDR and 46 % using LET), suggesting that 40 mites or possibly more can be identified per patient, from only two assessed eyelashes per eyelid by LET.

Conversely, epilation and light microscopic inspection of heavily infested lashes (confirmed by LET) frequently revealed low numbers of mites or no mites at all. The potentially repeated clinical procedure involving epilation of two lashes per eyelid may be undesirable, both with respect to cosmesis and comfort. In contrast, participants noted that CDR and LET were essentially imperceptible in sensation. The need for a laboratory microscope (with at least x100 magnification) limits the integration of epilation as a technique for *Demodex* diagnosis into routine clinical practice. The ability to accurately distinguish mites from epilated lashes may also rely on the quality and the settings of the laboratory microscope used, yet the make, model or magnification of the microscope used in previous studies has often not been declared. It is of note that the clinical quantification of mites with the proposed *in situ* techniques using widely available clinical biomicroscopes (at 40x

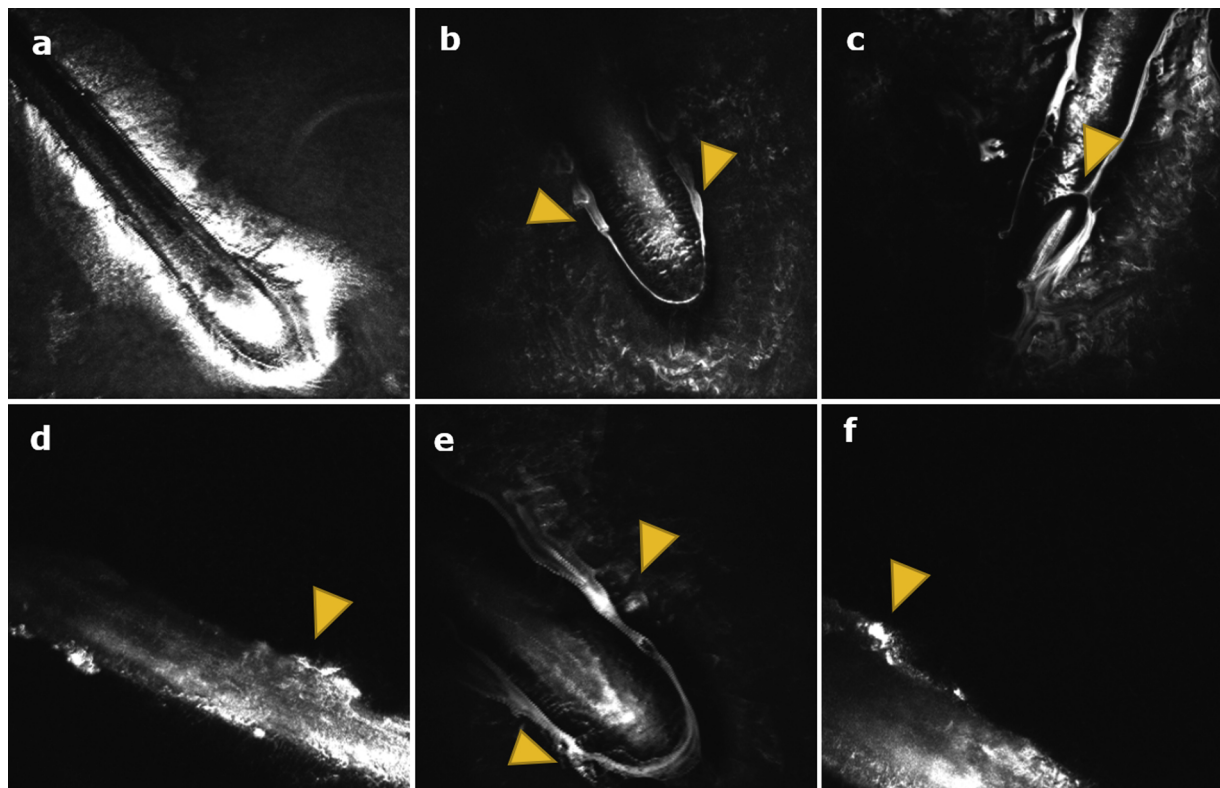


Fig. 4. IVCM of eyelash follicle (a) and presumed, unconfirmed *Demodex* (b) – (f) (arrows).

magnification), promotes image quality that is superior to that achievable digitally.

The rotation technique originally proposed by Mastrota has not previously been directly compared with the eyelash epilation technique. Prominent CD were noted to often obscure mite tails creating difficulty in distinguishing mite tails from collarette “shards” that might be generated during eyelash rotation. The present study showed that removing the CD (Video 1), along with excess cornified tissue surrounding the eyelash base and often fused to the epidermis, aided the differentiation of mite tails from surrounding features. Indeed, CDR alone revealed almost twice as many mites as ROT alone.

A second modification to the technique proposed by Mastrota [13] relates to the dynamics of lash manipulation. Mastrota likened the rotation of the eyelash within the follicle to that of a spatula used around the inside of a bowl, scraping around the inner perimeter of the eyelash follicle and “churning up” *Demodex* mites from within the follicle. However, it was noted in the current study that rotation of the central eyelash against the walls of the follicle, exerted pressure that risked forcing mites deeper into the follicle, particularly as the mite body is mostly buried in the follicle and only the tail tip protrudes. In contrast, lateral tension on the eyelash caused the eyelash orifice to elongate and the mite tails to fan out in the opposite direction. Large numbers of mites were thus revealed. These were found to mostly remain at the follicle opening during lash manipulation but occasionally would be expelled from the follicle by the lash manipulation to be distributed onto the surrounding lashes and lid margin (Fig. 1). Furthermore, in contrast to the ROT method in which retaining focus at high magnification presented challenges, LET combined with CDR to expose the eyelash insertion point into the lid margin allowed for more stable imaging and thus easier observation within the shallow depth of field of 40x biomicroscope magnification.

IVCM has been proposed as an efficient and reliable tool for the diagnosis of eyelid mite infestation. As described in early dermatological research [22], the hairless sebaceous glands of the face lend themselves to evaluation by this method. However, the eyelid shape together with the projection of eyelashes precludes the targeting of specific eyelashes and consistent imaging of the eyelash follicles, due to the shape of the TomoCap. In assessing over 400 images, *Demodex* mites could not be convincingly differentiated from adjacent features, such as CD and follicle irregularities (Fig. 4, Video 3). Even when isolated (in gel), mites appear difficult to distinguish, particularly if the mite aligns with the z-axis of the instrument. It may be possible that more extensive IVCM experience might afford more consistent eyelash follicle imaging to allow more reliable determination of *Demodex* presence, but the challenges experienced in the current study and the time consuming and costly nature of the assessment necessarily limit the diagnostic utility of IVCM for ocular *Demodex*.

This study is not without limitations. Mites residing deep within the follicle, as well as eggs, larvae and mites embedded in the removed collarette, may have been overlooked with CDR and LET techniques, resulting in an underestimated mite yield.

Currently the benefits that CDR might bear in itself, either as a prophylactic or therapeutic measure, are unknown. Fusion of the collarettes with adjacent epidermal tissue may form a seal, that isolates the eyelash follicle, and restricts access of anti-demodectic agents, such as tea tree oil-based products, delivered as therapeutic agents to the follicle. It is not inconceivable that removal of CD could facilitate such access and promote enhanced therapeutic effects.

To conclude, the cylindrical dandruff removal and lateral eyelash traction techniques confirm and complement the technique described by Mastrota, and offer an accessible, less invasive and more clinically

viable method for the assessment of *Demodex*, than eyelash epilation. Further studies evaluating the diagnostic sensitivity and specificity in age and sex matched groups across a range of infestation levels are warranted. In the meantime, however, this diagnostic tool demonstrates the potential to increase reliability in confirming *Demodex* presence, both in patient care, and in clinical studies and therapeutic trials. The fine manipulation of forceps and lashes under high-magnification observation proved to be a rapidly acquired skill for an eye care practitioner with biomicroscopy experience, while the ability to inspect many lashes efficiently and painlessly, suggests that CDR and LET can be easily integrated into standard clinical practice.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.clae.2019.11.009>.

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