In vivo confocal microscopy as a novel and reliable tool for the diagnosis of *Demodex* eyelid infestation

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ABSTRACT

Aims *Demodex* mites are implicated in several ocular surface diseases such as blepharitis, ocular rosacea and dry eye syndrome. *Demodex* eyelid infestation is classically diagnosed by analysing depilated eyelashes under the light microscope. The use of *in vivo* confocal microscopy (IVCM) could be an easy way to improve its diagnosis. The ability of IVCM to identify *Demodex* was evaluated and compared with the classic depilation method.

Methods Eight healthy subjects, 22 patients with dry eye syndrome without anterior blepharitis and 18 patients with anterior blepharitis were examined using lower eyelid IVCM (lash follicles and meibomian glands (MGs)). Twenty-five of the 48 subjects underwent both an IVCM examination and classic depilation to compare the two methods. *Ex vivo Demodex* obtained from lash depilation were also analysed using the confocal microscope.

Results IVCM found 100% of the mite infestations among patients with anterior blepharitis, 60% among dry eye patients without blepharitis and 12% in healthy subjects, whereas the depilation technique found 100%, 50% and 0%, respectively. *Demodex brevis* and *Demodex* larvae inside the lash follicles were better detected by IVCM. In symptomatic patients, the *Demodex* infestation was often associated with MG dysfunction, which was better characterised using IVCM in symptomatic patients (60% and 40% of meibomianitis and gland fibrosis, respectively).

Conclusions IVCM is an efficient and reliable tool for the diagnosis of eyelid mite infestation and may also provide an evaluation of MGs.

INTRODUCTION

The ectoparasite *Demodex* is the most common parasite in humans. It can be found in the eyelids, cilia, meibomian glands (MG), face and external ear tract. These mites are transparent, elongated in shape and divided into head-neck and body-tail parts, with eight short legs attached to the anterior body segment.[1] There are many species of *Demodex*, but only *Demodex folliculorum* and *Demodex brevis* are found on the human body.[2] *D folliculorum* lives in the lash follicles and measures 0.35–0.4 mm in length and *D brevis* lives deep in the MGs and the sebaceous glands of the lash, measuring 0.15–0.2 mm in length.[3] Because of their anatomical features, the eyelids are not accessible to routine cleansing hygiene, providing a favourable environment for *Demodex* mites to spread and flourish.[4] Although their pathogenic role remains unclear, previous studies have reported the important relationship between the existence of *Demodex* and blepharitis.[5] Their role in the modulation of the immune reactivity of the follicle and the fact that *Demodex* secretions function as a vector for bacteria could result in the development of blepharitis and dry eye syndrome.[6] Consequently, an accurate and reproducible method to quantify mite infestation of the eyelids would be useful for exploring these pathologies. However, the diagnosis of *Demodex* infestation remains difficult, due to the lack of specific symptoms or clinical signs and the need to collect eyelashes for light microscopy analysis.

Recently, *in vivo* confocal microscopy (IVCM) has been used for the diagnosis of *Demodex* skin infection by dermatologists.[7] Axial views of *D folliculorum* were clearly demonstrated in hair follicles. Indeed, *Demodex* have been implicated in several skin diseases such as rosacea, papulopustular eruptions and perioral dermatitis,[8] and IVCM has been presented as a useful tool to precisely quantify the mites in these diseases.[9] Without any special preparation, IVCM is also a modern imaging tool in ophthalmology that allows rapid visualisation of the eye surface with a nearly histological resolution. It has become an effective technique for the evaluation of corneal diseases[10 11] and conjunctiva, by evaluating goblet cell loss,[12 13] and the eyelids, especially for detecting MG dysfunction (MGD).[14 15] However, the use of IVCM for the analysis of peripheral structures such as the conjunctiva or the eyelids remains more challenging than the evaluation of the cornea due to its location and the high reflectivity of substantia propria.[16]

The purpose of the present study was, therefore, to use IVCM to investigate the *ex vivo* and *in vivo* characteristics of the two *Demodex* species, namely *D folliculorum* and *D brevis* and to quantify the mite infestation rate. To better understand its relationship with MGD, the presence of an infection of the meibomian orifices and meibomianitis or fibrosis was analysed.

PATIENTS AND METHODS

Patients

Eighteen patients (aged 45–85 years) suffering from anterior blepharitis (with cylindrical dandruff) and 22 patients (aged 32–80 years) suffering from dry eye syndrome (ocular surface disease index score >25, mild to severe conjunctival injection and tear film break-up time <10 s) without identifiable anterior blepharitis (patients suffering from ocular rosacea or MG disorders, Sjögren syndrome and

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atopic conjunctivitis) were examined with IVCM (one or both eyes). Those patients who had a history of Stevens–Johnson syndrome; chemical, thermal or radiation injury; keratoconus; a history of ocular surgery; or contact lens or drug use that would alter the ocular surface were excluded. Diagnosis of MGD is based on morphological features of the gland acini and duct orifices, presence of orifice plugging and thickening or absence of expressed excreta. Twenty-five underwent eyelid lash depilation (7–11 lashes per eye) followed by an optical microscopy examination (standard light microscope, ×10 or ×40). Eight young control subjects (aged 26–33 years) without clinical symptoms were also examined using the same methodology (table 1). This study was conducted at the Center of Clinical Investigations (CIC 503) at the Quinze-Vingts National Eye Center, Paris, France, with the approval of the Institutional Review Board of Saint-Antoine University Hospital (CPP-Ile de France 5, national agreement 10793). All the patients signed informed consent at the inclusion.

In vivo analysis of *Demodex* by IVCM

In vivo laser confocal microscopy was performed on all subjects with the Rostock Corneal Module Heidelberg Retina Tomograph II (HRTII Cornea Module; Heidelberg Engineering GmbH, Dossenheim, Germany). After an examiner asked the patient to look straight into a pointer light source, the centre of the Tomo-Cap was applanated onto the lower eyelid. The thumb of the examiner could change the exposure by pressing on the lower eyelid. The lower eyelid of all eyes was evaluated with IVCM (from temporal to nasal), allowing the examination of approximately 10 lashes and their follicles. Focal distance was modified to evaluate the whole follicle and lash root, and every suspected image of mites was recorded. Second, the image was interpreted by two masked ophthalmologists to distinguish between mites, lash and dandruff. Then the MGs were analysed. Four images were recorded in the temporal, medial and nasal eyelid. Meibomianitis was recognised by the presence of inflammatory cells[15] in the MG epithelium detected as hyper-reflectivity patterns between epithelial cells and interstice inhomogeneity (i.e. inflammatory cell density, ICD). The lumen was usually pathological with secretion reflectivity. Meibomian gland atrophy or fibrosis, as described by Villani,[15] was diagnosed by an acinar wall homogeneous hyper-reflectivity and decreased MG (MG) acinar density and size. Some patients had an IVCM cheek examination focused on the hair follicle, especially those patients suffering from rosacea. Usually, the entire process for both eyes required around 15 min with no discomfort for the patient.

Laboratory sample examination

Lash depilation was quickly performed after IVCM analysis as a standard technique. The average number of lashes was eight for each eye (range 7–11). Optical microscopy revealed the numbers of mites per lash (figure 1).

Ex vivo analysis of *Demodex* by IVCM

Demodex was also imaged with IVCM *ex vivo* in two patients suffering from dry eye syndrome. After depilation, the lashes were placed on slides and were examined under optical microscopy to confirm the presence of *D folliculorum*. Then the same slide was evaluated with IVCM (HRTII). The characteristics of *Demodex* were described by analysing the images and video samples.

RESULTS

Describing *in vivo* characteristics of *Demodex* in patients using IVCM

Different sections of mites were imaged: transversal, oblique and longitudinal, which was the most common. The distance between the lash follicle and MG was visible, as was meibomianitis (dendritic cells in the MG epithelium) next to the follicles.

D folliculorum were observed in various situations and in varying numbers: inside the follicle (figure 1A), inside the follicle next to MGs (figure 1B), between two eyelashes (figure 1C), at the bottom of a follicle (figure 1D) and in contact with the follicle papilla (figure 1E). Dead *D folliculorum* were observed attached to the base of the lash: no motility and non-typical *Demodex* images were interpreted to indicate death (figure 1F). When the number of *Demodex* was high, the reaction of lash follicles and secondary inflammation was also high. We noted follicle distension and epithelium hyper-reflectivity, which were more frequent with a high rate of infestation (anterior blepharitis and rosacea).

Suspected adult *D brevis* were seen at the very bottom of the follicle (figure 2A) or inside the MG meatus, usually one or two mites causing gland obstruction and reactional epithelial proliferation. We observed MG infection in eight cases in symptomatic patients as well as inflammatory cell infiltration such as hyper-reflective patterns around the orifices. *D brevis* appeared smaller with a larger prosoma (cephalothorax) than adult *D folliculorum* (around 250 versus 350 μm).

Table 1 Demographics, clinical data and patient examination

	Patients with anterior blepharitis n=18	Dry eye syndrome without cylindrical dandruffs n=22	Healthy subjects n=8
Age (median and range, years)	64 (45–85)	65 (32–80)	28 (26–33)
Gender, M/F	8/10	9/13	4/4
Diseases	Cylindrical dandruff around eyelash roots n=18 (100%) Dry eye syndrome n=10 (55%)	MGD n=14 (63%) MGD and rosacea n=11 (50%) Atopic conjunctivitis n=4 (18%) Sjögren disease n=4 (18%)	No dry eye No MGD
Lashes IVCM (%)	n=18 (100)	n=22 (100)	n=8 (100)
MG IVCM (%)	n=18 (100)	n=22 (100)	n=8 (100)
Cheek IVCM	n=8	n=8	n=4
Optical microscopy (%)	n=12 (66)	n=10 (45)	n=3 (37)

IVCM, *in vivo* confocal microscopy; MG, meibomian gland; MGD, meibomian gland dysfunction.

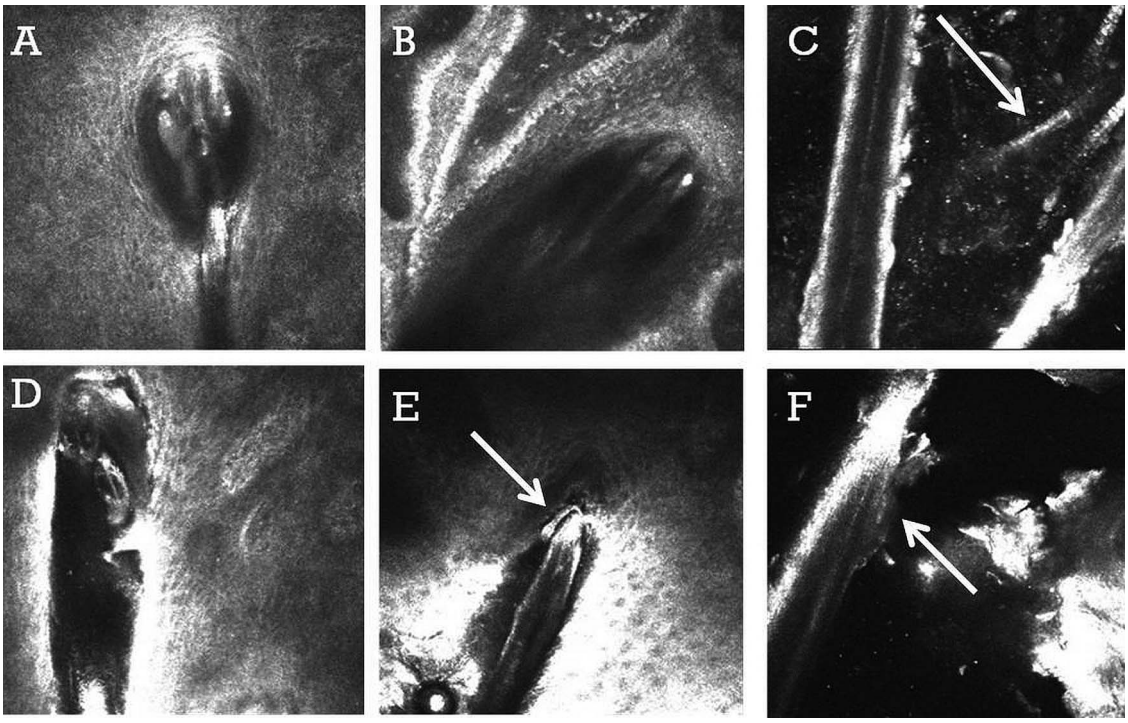

Figure 1 Presence of one or several *Demodex* in different anatomical structures. (A) Two *Demodex brevis* inside the follicle. (B) Three *D brevis* inside the follicle next to meibomian glands (MG) with hyper-reflectivity of the MG epithelium). (C) Three *Demodex folliculorum* between two eyelashes, arrow. (D) Three *D brevis* at the bottom of a follicle. (E) *Demodex* larva binding to the follicle's papilla, which could lead to lash instability, arrow. (F) Dead *D folliculorum* attached to the base of the lash, arrow (images: 400 μm×400 μm).

Discriminating *D brevis* from *Demodex* larvae was difficult. Larvae and protonymphs were imaged in the follicle in a cluster pattern (usually four to seven) measuring around 100 μm (figure 2B, C). *Demodex* larvae were observed in 31 cases (100% and 60% of patients with anterior blepharitis and the dry eye without anterior blepharitis, respectively) inside the follicles. No eggs were seen because of their small size (around 6 μm).

Comparisons between IVCM and the classic depilation/optical microscopy method for *Demodex* diagnosis

The presence or absence of mites was evaluated with both methods in 25 cases (12 patients with anterior blepharitis, 10 dry eye patients without anterior blepharitis and three healthy subjects) (table 2). Moreover, in cases of mite infestation, we distinguished a low infestation rate (≤3 *Demodex* per lash or less than eight *Demodex* in one eye) and a high infestation rate (>3 *Demodex* per lash or more than eight *Demodex* in one eye). With IVCM, more lash follicles could be examined (usually 10–15 on the lower eyelid) with the presence of *D brevis* and *Demodex* larvae, whereas the depilation technique did not allow an examination of the whole follicle, thus failing to diagnose these mite forms (0 specimens positive to *D brevis* and *Demodex* larvae).

All patients with anterior blepharitis had mite infestation. With IVCM, 18 out of 18 (100%) were found positive with a

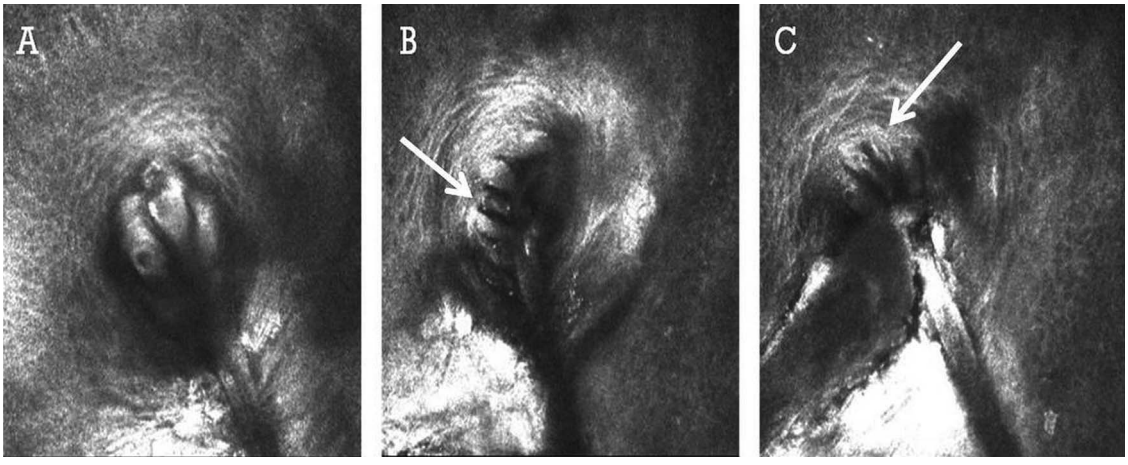

Figure 2 Infestation of eyelash follicles and secondary inflammation. (A) Three *Demodex*. (B) Six *Demodex* larvae with lashes, arrow. (C) Five *Demodex* larvae buried in the follicle, arrow, two lashes and one *Demodex folliculorum* along the base of one lash. (images: 400 μm×400 μm).

Table 2 Comparison of the classic depilation method with IVCM diagnosis in symptomatic patients and healthy subjects

	Patients with anterior blepharitis (n=18)	Dry eye without cylindrical dandruff (n=22)	Healthy subjects n=8
Diagnosis using depilation technique			
Low rate of mite infestation	5/12	5/10	0/3
High rate of mite infestation	7/12	2/10	0/3
Demodex folliculorum	12/12	5/10	0/3
Larvae	0/12	0/10	0/3
Total	12/12	5/10	0/3
Diagnosis using IVCM			
Low rate of mite infestation	0/18	7/22	1/8
High rate of mite infestation	18/18	6/22	0/8
D folliculorum	16/18	6/22	0/8
larvae or *Demodex brevis**	18/18	13/22	1/8
Total	18/18	13/22	1/8

*Demodex larvae and *D brevis* are grouped because they look too similar to distinguish.
IVCM, *in vivo* confocal microscopy.

high infestation rate; with the original depilation technique we found 12 out of 12 (100%) cases of infestation (seven with a high and five with a low infestation rate) (table 2).

As for the dry eye patients without anterior blepharitis (Sjögren disease, ocular rosacea or other causes) IVCM showed 13 of 22 had mite infestation (seven with low and six with high infestation). Rosacea was a risk factor because of the six with a high infestation rate, four had ocular rosacea. Patients with Sjögren syndrome and atopic conjunctivitis usually had no or mild infestation (table 3). Ten patients had undergone both examinations (three had no infestation with both exams, four had low infestation with both exams, one had low infestation with the depilation technique and a higher infestation rate with IVCM and two had high infestation at both exams).

In the young asymptomatic patients, none had mite infestation with the original technique (positivity 0/3), whereas, IVCM diagnosed a low infestation rate (positivity 1/8) in one subject.

MG disease diagnosis

Among patients with anterior blepharitis, eight (44%) had meibomianitis and 10 (56%) had MG fibrosis; among the dry eye patients without anterior blepharitis, 16 (72%) had meibomianitis and six (28%) had MG fibrosis, whereas no pathological pattern was detected among asymptomatic patients (table 4, figure 3). The mean ICD of four images was measured with the internal software.

For the first time, we also performed the same IVCM method on the patient's cheek (skin overlying the maxillary sinus), and *Demodex* were observed inside the hair follicle in 10 patients. Six suffered from rosacea and four from anterior blepharitis with cylindrical dandruff (figure 4). The cheek examination was not systematic, and only 20 patients were analysed.

Table 3 infection grade depending on ocular diseases in dry eye syndrome without anterior blepharitis

Type of disease	No infection	Mild infection	Severe infection
MGD±ocular rosacea	3	5	6
Sjögren syndrome	3	1	0
Other causes	3	1	0
Total	9	7	6

MGD, meibomian gland disorder.

Describing *ex vivo* characteristics of *Demodex* using IVCM

The prosoma was visible with eight short legs. If the *Demodex* was alive, the head and legs were moving. The *Demodex* body was observed as an elongated structure measuring approximately 350 μm in length. The abdomen reflectivity was heterogeneous and striated (figure 5A–D). Neither larvae nor *D brevis* were detected at either examination.

DISCUSSION

This study investigated the use of IVCM as a novel and reliable tool for the diagnosis of *Demodex* eyelid infestation. It is a suitable candidate for the rapid detection of micro-organisms living in the eyelid and also for the exploration of nearby eyelid structures.

In the present study, the sensitivity of IVCM *Demodex* diagnosis seems similar to the original technique of *ex vivo* microscopic examination after depilation, and even better for detecting low grades of infestation. IVCM allows a complete *in vivo* examination of the follicle including the detection of *D brevis* and *Demodex* larvae, unlike depilation of the eyelashes. Gao *et al*[2] found five *D brevis* mites and zero larvae among 422 *Demodex*-positive specimens, which is a low rate. This is likely due to the position of *D brevis* and *Demodex* larvae, which

Table 4 Comparison of meibomian gland examination with *in vivo* confocal microscopy in symptomatic patients and healthy subjects

	Patients with anterior blepharitis (n=1)	Dry eye without cylindrical dandruff (n=22)	Healthy patients n=8
Meibomianitis	8/18	16/22	0/8
Mean ICD (cells/mm²)	265 (151–536)	258 (8–456)	No ICD
MG fibrosis	10/18	6/22	0/8
MG meatus infection	7/18	4/22	0/8

ICD, inflammatory cell density; results are reported as mean (range); MG, meibomian gland.

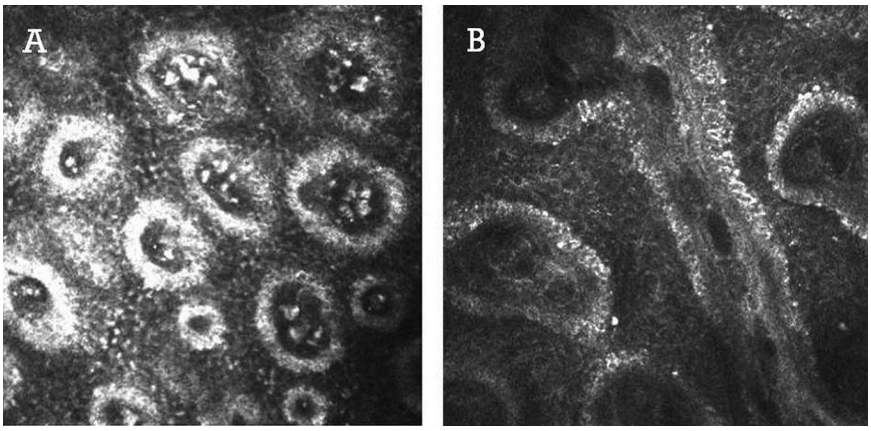

Figure 3 Meibomianitis. (A and B) Meibomian glands with meibomianitis, with high reflectivity of gland epithelium, secondary to inflammatory cell infiltration, and heterogeneous reflectivity of the gland lumen. (images: 400 μm×400 μm).

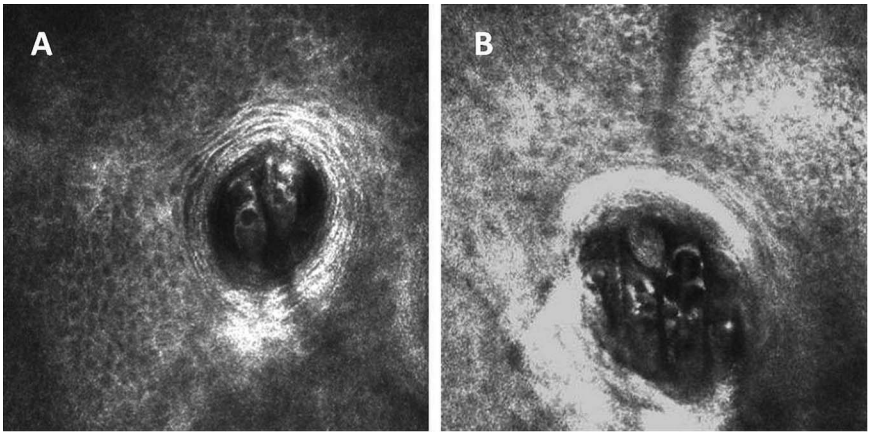

Figure 4 Hair follicle infection on cheek skin. (A and B) Cluster of *Demodex*, especially the episthosoma. (images: 400 μm×400 μm).

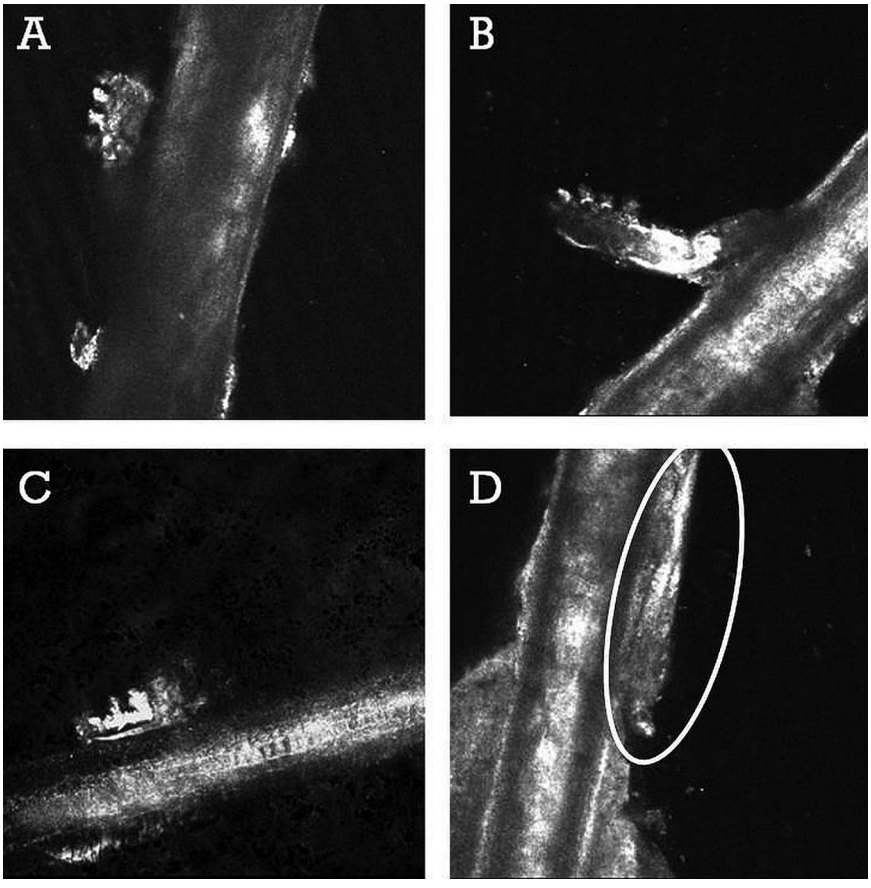

Figure 5 *Demodex folliculorum ex vivo* on an eyelash using confocal microscopy. (A) Longitudinal view of a *Demodex*. (B) Bending of a *Demodex* in longitudinal view. (C) *Demodex* head. (D) *Demodex* body (white circle). (images: 400 μm×400 μm).

burrow deep into sebaceous glands looking for sebum.[8] The larvae could migrate to the papilla follicles and lead to lash instability and madarosis, disturbing epilation sensitivity. Another advantage is the diagnosis of infestation in complete madarosis patients (chemotherapy).

Additionally, the value of IVCM is to establish the presence of *Demodex*, because the MGs are also visible just beneath the follicle, which allows a detailed examination of the eyelid. The decision to treat is thus more reliable. For example, a low presence of *Demodex* without MGD visible in IVCM and no or few clinical symptoms would not need treatment but a simple follow-up.

We also investigated the *Demodex* involvement in dry eye patients without anterior blepharitis. Using IVCM, 13 out of 22 had mite infestation. Rosacea could be considered a risk factor of *Demodex* infection rather than Sjögren syndrome or atopic conjunctivitis syndrome. In fact, four high-rate infection patients out of six suffered from rosacea. The relatively high level of mite infestation in dry eye patients revealed a possible role of *Demodex* in dry eye, but it is impossible to determine if *Demodex* infestation is the consequence of dry eye, possibly through the induction of inflammation and the secondary obstruction of MG orifices,[17] or if MGD was the cause of dry eye in these cases.

The limits of IVCM are the problems distinguishing the two *Demodex* species and between *D brevis* and *Demodex* larvae. Further studies are needed to improve IVCM-eyelid semiology because it is not known whether both species are equally implicated in the pathogenesis of eyelid disorders. It should be acknowledged that the examination by IVCM for *Demodex* could also have false-positive results. There is a risk of mistaking a mite for a thin lash or dandruff. However, the eyelash had some characteristic features. The risk of false-negative results also exists because total examination of the eyelid was not possible. There are 75 lashes on two or three rows and the exam would have been too long. Usually only 10–20 lashes are examined, which is more than the depilation technique with only 7–11 eyelashes usually examined. We also compared the IVCM diagnosis with the classic depilation method. In fact, the sensitivity of depilation diagnosis is unknown. Lashes with cylindrical dandruff are 10 times more likely to contain *Demodex* mites, which means that the choice of lashes to be depilated may influence the diagnosis of *Demodex* infestation, further impairing the reliability of the conventional technique.[2]

IVCM also allows frequent exams, and easy follow-up is possible over time. The advantage is that it is non-invasive, and a monthly examination can easily be carried out to ensure mite eradication, compared with depilation, which is far from being painless and cannot be repeatedly proposed. Kojima *et al*[18] showed that this tool effectively detects the decrease of mites after 6 weeks of tea tree oil treatment. The relation between eyelid and cheek skin infestation should be studied, especially among patients with rosacea. IVCM could be particularly advantageous in centres without access to a well-equipped laboratory for *Demodex* diagnosis.

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