Persian J. Acarol., 2021, Vol. 10, No. 4, pp. 491–499.
https://doi.org/10.22073/pja.v10i4.69561
Journal homepage: http://www.biotaxa.org/pja

Article

Enhancing survival of *Demodex folliculorum* (Acari: Demodecidae) under *in vitro* condition: Effect of temperature and culture media

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ABSTRACT

Demodex spp. are ectoparasites that live on mammal's skin. *Demodex folliculorum* Simon is important in the pathogenesis of blepharitis and other inflammatory skin diseases; however, the *in vitro* culture has not been achieved yet. In this study, the effect of temperature and culture medium on the survival of *D. folliculorum* was determined. Adult mites were obtained directly from eyelashes of individuals with a clinical diagnosis of *Demodex* blepharitis. Temperature ranges from 15–25 °C, and three culture media (RPMI-1640, RPMI-1640 supplemented with human serum, and RPMI-1640 supplemented with fetal bovine serum) were tested. Results showed significant differences in the survival time of *D. folliculorum* among different treatments. The maximum survival of mite was 15.20 ± 1.03 days with RPMI-1640 supplemented with human serum at 20 °C. This result is the longest reported survival time of *D. folliculorum* under *in vitro* maintenance conditions. This research presents a refinement of the methods previously reported to develop an *in vitro* culture of *D. folliculorum.*

KEYWORDS: Blepharitis; culture conditions; *demodicosis*; ectoparasitic mites; *in vitro* maintenance.

PAPER INFO.: Received: 18 June 2021, Accepted: 12 August 2021, Published: 15 October 2021

INTRODUCTION

Demodex is a genus of ectoparasite mites that live in mammal pilosebaceous follicles and sebaceous glands. These mites feed of follicular and glandular epithelial cells causing direct damage to the cell walls (Lacey *et al*. 2016; Litwin *et al*. 2017). *Demodex folliculorum* Simon and *Demodex brevis* Akbulatova, are the only two mite species that infest humans. *Demodex folliculorum* lives alone or in groups on hair follicles. In contrast, *D. brevis* usually lives alone in sebaceous and meibomian glands (Thoemmes *et al*. 2014).

Epidemiological studies report Demodex infestation in individuals of all races and geographic areas and no preference in sex (Wesolowska *et al*. 2014). Besides, the infestation has been correlated

How to cite: Maldonado, H., Mendoza, D., Garavito, G., Lizarazo, M., Escaf, L. & Egea, E. (2021) Enhancing survival of *Demodex folliculorum* (Acari: Demodecidae) under *in vitro* condition: Effect of temperature and culture media. *Persian Journal of Acarology*, 10(4): 491–499.

significantly with increasing age (Mongi *et al*. 2018; Demirkazık and Koltaş 2020). In addition, clinical studies have shown a close relationship between the *Demodex* infestation and inflammatory skin diseases such as rosacea, perioral dermatitis (Aktaş Karabay and Aksu Çerman 2020), blepharitis (Kabataş *et al*. 2017), and other external ocular lesions (Nicholls *et al*. 2017). For example, high infestation with *D. folliculorum* has been associated with follicular distention and loose and misdirect lashes; also, with micro-abrasions, epithelial hyperplasia, and reactive hyperkeratinization around the base of the lashes, and cylindrical dandruff forming (Luo *et al*. 2017; Zhong *et al*. 2019).

Although many aspects of the cause-effect relationship between *Demodex* infestation and blepharitis are still unknown, recent research indicates that alterations in the periocular microbiota equilibrium would increase the colonization of more virulent forms of *Demodex* associated with endosymbiotic pathogens (Chen and Plewig 2015; Tatu *et al*. 2016; Yan *et al*. 2020). In this sense, a study in patients with chronic blepharitis showed that *D. folliculorum* is a carrier of the pathogenic bacteria *Bacillus oleronius* (Szkaradkiewicz *et al.* 2012); while *D. brevis* has been associated with Streptococci and Staphylococci (Liu *et al*. 2010).

The development of *in vitro* cultures of *Demodex* spp. is one of the main challenges in experimental acarology (Litwin *et al*. 2017). These cultures are necessary for studying the basic biology and host-parasite interactions. In addition, cultures would allow identifying the life cycle stages that could be susceptible to treatment without affecting the normal microbiological equilibrium of the skin. To date, cultures of *Demodex* spp. have not been achieved yet, due to the low survival of these mites and the difficulty in reproducing them under ex vivo conditions. One can find studies that have shown that the *in vitro* viability of *D. folliculorum* is affected by different physical and nutritional parameters (Zhao *et al*. 2009, 2011). Therefore, the objective of the present study was to establish the best conditions of temperature and nutritive culture medium for the survival of *D. folliculorum* under *in vitro* conditions.

MATERIALS AND METHODS

Mites

Demodex mites were obtained from adult individuals diagnosed with blepharitis with cylindrical dandruff who went for professional medical consultation in Barranquilla (Colombia, South America). Blepharitis with *Demodex* infestation was diagnosed using an examination under a slit lamp (Carl Zeiss, SL 130) (Fig. 1).

Two eyelashes from each eyelid were taken from every patient using watchmaker's forceps (Rumex®). *Demodex* mites were carefully removed from the eyelashes with a dissection needle under the light of an optic microscope (Nikon model TMS No 211850). They were identified using the morphological characteristics of *Demodex* species described by Desch and Nutting (1972). *Demodex folliculorum* mites were deposited (10 mites/well) in polystyrene sterile six-well cell culture plates (Millipore Ref. no.703001), containing 3 ml of culture medium per well.

Culture medium

RPMI 1640 supplemented with L-glutamine and HEPES buffer (Gibco, Ref. 11875093), RPMI 1640 enriched with Fetal Bovine Serum (FBS) (LANG & M, Ref. 12657029), and RPMI 1640 enriched with normal Human Serum (HS) (Millipore, Cat No S1-100ML) were used in this study. Penicillin and streptomycin (100 units/mL, Gibco-Invitrogen, Ref. 15140-122) were added to each medium to prevent bacterial contamination of cultures due to their effective combined action against gram-positive and gram-negative bacteria. Media were chosen according to the physiology of *D. folliculorum* and the results of a previous study (Zhao *et al.* 2011).

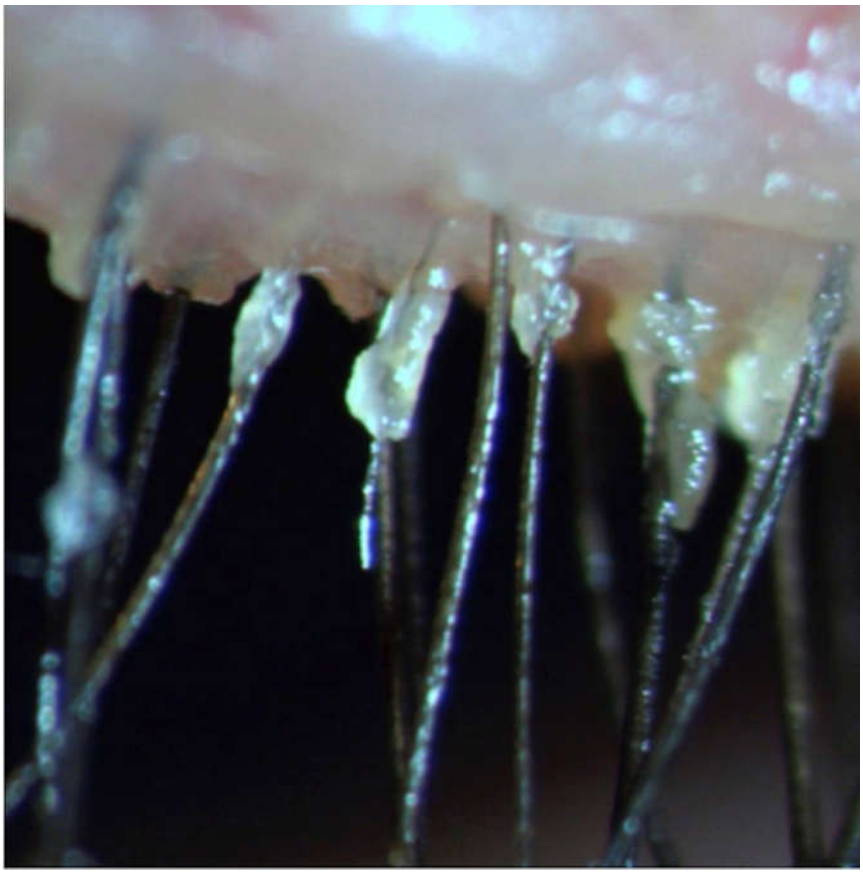

Figure 1. Photograph of the eyelids of a patient with cylindrical dandruff attached to the eyelid margin and around the eyelash base.

Survival test

A factorial experimental design 32 (Table 1) was applied to evaluate the main effects and interactions among temperature and culture media over the mite's survival time. The independent variables (Factors) were:

- Temperature: 15, 20, and 25 °C
- Culture medium: RPMI 1640, RPMI 1640 – FBS, and RPMI 1640- HS

Table 1. Experimental conditions of the survival test.

Treatment	Factors	
	Temperature (°C)	Culture media
1	15	RPMI 1640
2	20	RPMI 1640
3	25	RPMI 1640
4	15	RPMI 1640-FBS
5	20	RPMI 1640-FBS
6	25	RPMI 1640-FBS
7	15	RPMI 1640-HS
8	20	RPMI 1640-HS
9	25	RPMI 1640-HS

Mite cultures were incubated in an environment and humidity proof chamber (SHEL LAB Model HC30-R) under conditions of darkness, humidity 90%, and a specific temperature for each treatment. Every 72 hours, 2 mL of fresh medium was added to the wells to make up for the volume lost due to evaporation. Mites were monitored using an optic microscope (40× objective) every 8 hours until all the mites had died. The time of death of mites was recorded. Lack of movement in appendices (chelicerae) or legs during a minute was taken as an indicator of death. A second observation was done 30 minutes later to verify the death of the mites. The results were expressed as mite's survival time. Each experimental condition was performed in duplicate.

Statistical analysis

The results of mite survival were presented as mean and standard deviation (SD). Differences among the means of different treatments were evaluated with a one-way analysis of variance (ANOVA) with a significance level of 0.05 %. The normal distribution of the residuals was checked using the Shapiro-Wilk test (W = 0.97, p-value = 1.03), and the homoscedasticity (equal variance) was determined with Levene's test. Later, a pairwise multiple comparisons test was made by Tukey's HSD (honestly significant difference) test. A p-value < 0.05 was considered statistically significant. All data analysis was made on SPSS statistical package version 21.0 for Windows (SPSS, Inc., Chicago). Plots were made in the free statistic software RStudio version 1.1.383.

Ethical considerations

The ethical committee of the participating institutions approved this study with a previous verification of compliance with international biomedical investigation norms. This study was adjusted to the Colombian normative regulation determined by the Law 84 of 1989; and the guidelines prescribed in resolution No. 008430 of 1993 issued by the Ministry of Health of Colombia. All patients signed a consent form after being informed of the biological sample collection.

RESULTS

Figure 2 shows *D. folliculorum* mites attached to the hair follicles of the eyelashes of individuals affected by *Demodex* blepharitis who were enrolled in this study. Most of the eyelashes studied had more than one viable mite (between 2–6), being identified four life stages: ovum, larva, nymphs, and adult. The cultures were carried out only with adult mites that showed high mobility after being separated from the eyelash.

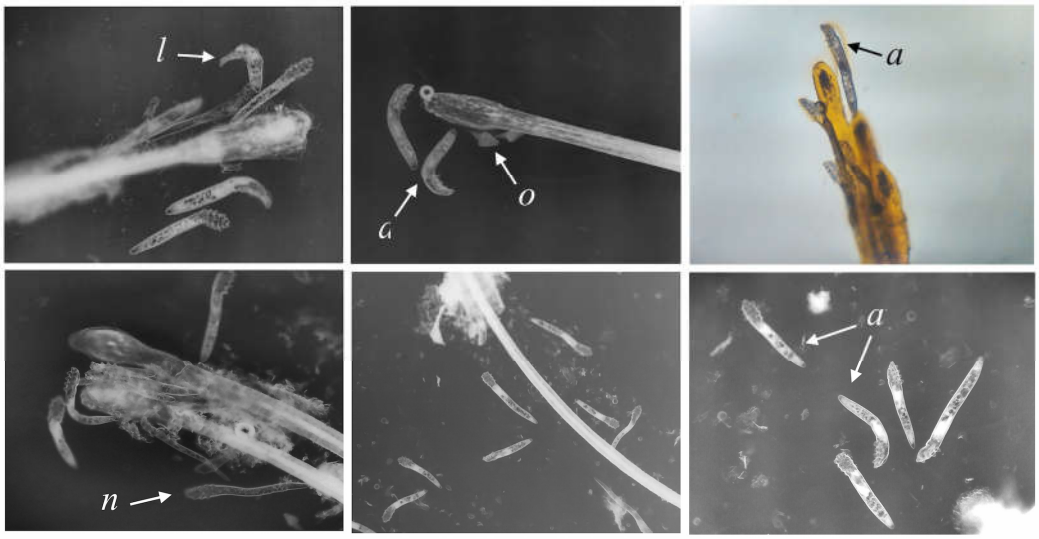

Figure 2. Microphotograph (40×) of *Demodex folliculorum* mites in the hair follicle of six individuals with a clinical diagnosis of blepharitis. Note the different life stages of the mites (*a* = adult; *l* = larva; *n* = nymph; *o* = ovum).

Survival time of *D. folliculorum* varied among the treatments (Table 2). The highest survival time of the mites was in RPMI-1640 enriched with HS medium, at 20 ℃ (Mean = 15.20 ± 1.32 days). On the contrary, at 15 ℃ and 25 ℃ temperatures, the mite's survival was significantly reduced, independent of the culture medium used. ANOVA showed a significant difference between the means of the treatments (*P-value* < 0.01). Post-hoc analysis by Tukey's HSD test showed that both temperature and culture medium significantly influence the *D. folliculorum* survival time. When survival times were compared at 15 ℃, no statistically significant difference was observed between different culture media (ANOVA, *P-value* = 0.51). However, when the mites were kept at 20 ℃ and 25 ℃, there was a significant statistical difference between the means of survival times (ANOVA, *P-value* at 20 ℃ = 2.7 e-09 and p-value at 25 ℃ = 3.42 e-03). These results suggest that at temperatures higher than 15 ℃, the culture medium composition significantly influences the survival of *D. folliculorum.*

Table 2. Survival time (days) of *Demodex folliculorum* at different temperatures and culture media.

Treatments		Survival time (days) (Mean ± SD)*
Culture media	**Temperature (℃)**	
RPMI 1640	15	7.30 ± 0.95^{de}
	20	9.30 ± 1.16^{c}
	25	7.40 ± 1.17^{de}
RPMI 1640 – HS	15	7.50 ± 0.85^{de}
	20	15.20 ± 1.32^{a}
	25	8.90 ± 0.99^{cd}
RPMI 1640 – FBS	15	7.00 ± 1.05^{e}
	20	12.10 ± 1.66^{b}
	25	8.80 ± 0.79^{cd}

* Data are given as means ± standard deviation; treatments sharing the same letter are not significantly different at 95% confidence.

DISCUSSION

To date, there are very few studies on the *in vitro* culture of *D. folliculorum*. However, previous publications reported that factors such as medium type, humidity, temperature, and medium pH significantly influence the survival of *D. folliculorum* and *D. brevis* in laboratory conditions (Chen 1985). In addition, Zhao *et al*. (2009) found an inverse correlation between the mites' survival and the 5–37 ℃ temperature range, with an optimum development temperature of 16–20 ℃ for both mite species. This result was similar to that obtained in our work, where 20 °C was the best condition for *D. folliculorum* survival for all three culture mediums tested under 90% humidity and darkness conditions.

Zhao *et al*. (2011) also evaluated the combined effect of temperature with different media on the viability of *D. folliculorum* and *D. brevis*, obtaining average survival times of less than 90 hours (3.75 days) when using human serum and a humidity of 98% at a temperature of optimal maintenance between 16–22 ℃. In addition, Shiels *et al.* (2019) demonstrated the survival of *D. folliculorum* of 2.5 to 3.5 days at 28 °C, after a storage period of 5 days at 4 °C. In our study, human serum was also the best medium for the survival of *D. folliculorum*; however, the maximum survival time was significantly longer (324 hours/15 days). It is considered possible that factors such as the mite collection methodology could influence their viability. In contrast to other studies where mites were collected with the cellophane tape method (Wu and Meng 1990), in the present study, the specimens

were separated directly from the patient's eyelashes, which reduced the physical damage and stress of mites. Furthermore, greater control over humidity (90%) and darkness could positively influence the results.

Other culture mediums reported in previous survival studies of *Demodex* are Dulbecco's Modified Eagle Medium (DMEM), Iscove's Modified Dulbecco's Media (IMDM), glycerin (50%, 70%), mineral oil, paraffin, and pork oil. Of these, only paraffin oil at 15 °C showed a maximum survival time related to the results obtained in this study (13–17 days) (Shufang *et al.* 2003). Notably, Zhao *et al.* (2011) also used paraffin oil (paroleine) to maintain *D. folliculorum* and *D. brevis*; however, their results differed from those reported by Shufang *et al.* (2003), with shorter survival times (80 hours/3.3 days) at 16–20 °C. Liquid paraffin oil is one of the most straightforward and most economical mediums for preserving microbial cultures. In the case of *D. folliculorum*, paraffin could act as maintenance support, helping protect the mites from the effect of some toxins. Still, it would scarcely provide essential nutrient support for the mites to survive for more extended periods. In contrast, mediums enriched with human serum could simulate better nutritional and physiological conditions to the *in vivo* mites because it contains primary metabolites (carbohydrates, lipids, and proteins), vitamins, minerals, hormones, and vital growth factors for its development.

Although it was possible to increase the life span of mites, none of our experiments showed mites' reproduction, which is essential for establishing cultures. Consequently, complementary protocols tending to improve the conditions of *in vitro* growth of *D. folliculorum* are necessary, especially the use of other substrates or growth supports. In the present investigation, only one type of support was used for the cultures (polystyrene plates); so, other alternatives that better simulate the natural microenvironment of *Demodex* spp. would need to be explored. Substrates such as cellulose pads, gelatin, paraffin, glass beads, and goat hair have been used in intent to culture different skin mites, such as the *Sarcoptes scabiei*, achieving a maximum survival time of 5 days on cellulose pads (Tarigan 1998). Another alternative suggested for further studies is the use of skin tissue substitutes or artificial skin. Recently, Japanese researchers developed a three-dimensional integumentary organ system, including follicles and sebaceous glands (Takagi *et al*. 2016). In the future, this type of tissue could be used as skin models for ex vivo cultures of *D. folliculorum.*

CONCLUSIONS

The *in vitro* culture of *D. folliculorum* represents a significant challenge due to the complexity of the mite's physiology. A significantly increased *in vitro* mite survival time was achieved in our study compared with previous studies. Because during the experiment, no indicators of growth or development of mites were observed, RPMI-HS would only be helpful as a means of maintenance of *D. folliculorum*. Prospects will be aimed at achieving *in vitro* culture models that keep the mites alive through various stages of life. Achieving this objective would allow more information and apply this new knowledge on mite biology and its relation to blepharitis and other human skin conditions.

Funding

This investigation was made possible with resources from the research project: Production and Evaluation of a Pharmaceutical Preparation for the Control and Prevention of Blepharitis and Keratoconjunctivitis caused by the *Demodex* sp. Ectoparasite, approved by “Ministerio de Ciencia, Tecnologia e Innovacion” in Colombia (Grant 436254832787).

ACKNOWLEDGEMENTS

The authors thank the nursing personnel at the “Clinica Oftalmológica del Caribe” and the patients that allowed us to obtain biological samples.

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افزایش زنده‌مانی *Demodex folliculorum* (Acari: Demodecidae) در شرایط درون شیشه‌ای: اثر دما و محیط کشت

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چکیده

گونه‌های *Demodex* انگل‌های خارجی‌اند که روی پوست پستانداران زندگی می‌کنند. گونهٔ *Demodex folliculorum* Simon در بیماریزایی بلفاریت و دیگر بیماری‌های التهابی پوست مهم است؛ با این حال، کشت درون شیشه‌ای هنوز انجام نشده است. در این بررسی، اثر دما و محیط کشت بر زنده‌مانی *D. folliculorum* تعیین شد. کنه‌های کامل به طور مستقیم از مژه‌های افراد با تشخیص بالینی بلفاریت دمودکس به دست آمدند. دامنهٔ دما بین ۱۵ تا ۲۵ درجهٔ سلسیوس بود و سه محیط کشت (RPMI-1640، RPMI-1640 با سرم انسان و RPMI-1640 با سرم جنین گاوی) مورد آزمایش قرار گرفت. نتایج تفاوت معنی‌داری را در زمان زنده‌مانی *D. folliculorum* در بین تیمارهای مختلف نشان داد. بیشینهٔ زنده‌مانی کنه ۱/۰۳ ± ۱۵/۲۰ روز با RPMI-1640 با سرم انسانی در ۲۰ درجهٔ سلسیوس بود. این نتیجه طولانی‌ترین مدت زنده ماندن *D. folliculorum* در شرایط نگهداری درون شیشه‌ای است. این پژوهش اصلاح روش‌هایی را ارایه می‌کند که پیش‌تر برای توسعهٔ کشت درون شیشه‌ای *D. folliculorum* گزارش شده‌اند.

واژگان کلیدی: بلفاریت؛ شرایط کشت؛ دمودیکوز؛ کنه‌های انگل خارجی؛ نگهداری درون شیشه‌ای.

اطلاعات مقاله: تاریخ دریافت: ۱۴۰۰/۳/۲۸، تاریخ پذیرش: ۱۴۰۰/۵/۲۱، تاریخ چاپ: ۱۴۰۰/۷/۲۳