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AUTOR: Ivan Ravera

DIRECTORES: Lluís Ferrer, Mar Bardagí, Laia Solano Gallego.

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Dr. Lluis Ferrer i Caubet, Dra. Mar Bardagí i Ametlla y Dra. Laia María Solano

Gallego, docentes del Departamento de Medicina y Cirugía Animales de la

Universidad Autónoma de Barcelona,

HACEN CONSTAR:

Que la memoria titulada "Deconstructing canine demodicosis" presentada por el

licenciado Ivan Ravera para optar al título de Doctor por la Universidad

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Bellaterra, el 23 de Septiembre de 2015.

Dr. Lluis Ferrer, Dra. Mar Bardagi,

Dra. Laia Solano Gallego

Directores de la tesis doctoral

Ivan Ravera

Doctorando

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1. ABBREVIATIONS

HLA Human Leukocyte Antigen

CanD Canine Demodicosis

DLA Dog Leucocyte Antigen

MHC Major Histocompatibility Complex

MDR-1Δ Multi Drug Resistance-1Δ gene

ABCB1-1Δ Adenosine triphosphate Binding Cassette Sub-Family B1-1Δ

TLR Toll-like Receptors

IL Interleukin

MyD-88 Myeloid Differentiation primary response gene-88

NF-κB Nuclear Factor kappa-light-chain-enhancer of activated B cells

TNF Tumor Necrosis Factor

SLIF Serum's Lymphocyte Immunoregulatory Factor

TGF- β Transforming Growth Factor β

PCR Polymerase Chain Reaction

CH Chitin synthase

bp **b**ase **p**air

DNA Deoxyribonucleic Acid

RNA Ribonucleic Acid

rDNA ribosomal Deoxyribonucleic Acid

rRNA ribosomal Ribonucleic Acid

mtDNA mitochondrial Deoxyribonucleic Acid

COI Cytochrome Oxidase I

Ig Immuno**g**lobulin

kDa **k**ilo **Da**ltons

Th1 T-helper 1

Th2 T-helper 2

PBS Phosphate Buffered Saline

SCAR Sequence Characterized Amplified Regions

RAPD Randomly Amplified Polymorphic DNA

2. SUMMARY

It is considered that *Demodex* mites are normal inhabitants of the mammalian skin. They have been adapted to live inside skin hair follicles and sebaceous glands of mammalian hosts. Demodicosis can be defined as an inflammatory skin disease characterized by the presence of *Demodex* mite overpopulation. It is considered that the cellular immune response is responsible for the control of mite population, while the role of the humoral and innate immune responses remains unknown. In domestic animals, the most severe form of demodicosis occurs in dogs. Consequently, canine demodicosis is the most studied disease produced by *Demodex* mites. Two canine *Demodex* species have been identified: *Demodex canis*, and *Demodex injai*, while a third species unofficially named *Demodex cornei*, has been proposed. Many studies have tried to report *Demodex* prevalence in healthy dogs by different methods.

In the present doctoral thesis, we described a real-time PCR technique to detect *Demodex* DNA (sequence of chitin synthase gene) in canine hair samples. This technique demonstrated the presence of *Demodex* mites in higher percentages than previous reports, suggesting that *Demodex* mites are present in all healthy dogs independent of age, sex, breed, or coat. Furthermore, *Demodex* populations were distributed in small numbers along the dog's body.

In order to analyze the phylogenetic relationships between the two canine *Demodex* species and the proposed third species, we amplified and sequenced a fragment of the mitochondrial 16S rDNA gene. Phylogenetic analysis revealed that *D. injai* is a different species from *D. canis*. In addition, it demonstrated that *D. cornei* is probably a morphological variant of *D. canis*. A conventional PCR for the specific detection of *D. injai* DNA was also developed and standardized. This technique demonstrated that *D. injai* is also a normal inhabitant of some dogs. Moreover, it suggested that in the majority of clinical canine demodicosis cases, an overgrowth of *D. injai* is unlikely.

Finally, to enlighten the field of the humoral response in canine demodicosis, a *D. canis* crude extract antigen was obtained and we demonstrated the presence of immunoglobulins G against several *D. canis* antigens in the sera of healthy dogs and in the sera of dogs with juvenile generalized demodicosis with and without secondary complicating pyoderma by western blot technique.

In conclusion, this doctoral thesis demonstrated that *Demodex* mites are normal inhabitant of the canine skin, they are present in the majority of dogs, and are distributed in very low numbers along all the haired skin. Furthermore, *Demodex injai* must be considered a different species from *D. canis*, and *D. cornei* is a probable morphological variant of *D. canis*. Healthy dogs and dogs with canine juvenile generalized demodicosis have an acquired humoral immune response against *Demodex* mites and present serum antibodies directed against several *Demodex canis* protein antigens.

RESUMEN

Los ácaros *Demodex* son habitantes normales de la piel de los mamíferos. Los mismos se han adaptado a vivir dentro de los folículos pilosos y las glándulas sebáceas de la piel de sus huéspedes mamíferos. La demodicosis puede ser definida como una enfermedad inflamatoria cutánea caracterizada por la presencia de una sobrepoblación de ácaros *Demodex*. Se considera que la respuesta inmunitaria celular es la responsable del control de la población de ácaros, mientras que los roles de las respuestas inmunitarias humoral e innata, son desconocidos. En los animales domésticos, la forma más grave de la demodicosis, la padecen los perros. En consecuencia, la demodicosis canina es la enfermedad más estudiada. Se han identificado dos especies de ácaros *Demodex* en perros: *Demodex canis* y *Demodex injai*; mientras se ha propuesto una tercera especie denominada *Demodex cornei*. Muchos estudios han tratado de documentar la prevalencia de ácaros *Demodex* en los perros sanos mediante diferentes métodos.

En la presente tesis doctoral, se describe una técnica de reacción en cadena de la polimerasa (PCR) a tiempo real para detectar ADN de *Demodex* (secuencia del gen de la quitina sintetasa) en pelos de perros. Ésta técnica demostró la presencia de ácaros *Demodex* en porcentajes más altos que en los estudios anteriores, sugiriendo que los ácaros *Demodex* están presentes en todos los perros sanos independientemente de la edad, sexo, raza o pelaje. Además, la técnica demuestra que las poblaciones de *Demodex* están distribuidas a lo largo del cuerpo de los perro en pequeño número.

Con el fin de establecer las relaciones filogenéticas entre las dos especies caninas y la propuesta tercera especie, amplificamos y secuenciamos un fragmento del ADN mitocondrial de la subunidad ribosomal 16S. El análisis filogenético demuestra que *D. injai* es una especie diferente de *D. canis* y que *D. cornei* es probablemente una variante morfológica de *D. canis*. Además, se desarrolló y estandarizó una técnica de PCR convencional para la detección específica de ADN de *D. injai*. Ésta técnica demostró que *D. injai* también es un habitante normal de la piel de algunos perros y que en la mayoría de los casos clínicos de demodicosis canina, la sobrepoblación de *D. injai* es infrecuente.

Finalmente, con el fin de ampliar los conocimientos de la respuesta humoral en la demodicosis canina, obtuvimos un extracto antigénico crudo de *D. canis* y demostramos, mediante una técnica de Western-blot, la presencia de inmunoglobulinas G contra diversos antígenos de *D. canis* en el suero de perros sanos, de perros con demodicosis juvenil generalizada con o sin infección cutánea secundaria.

En conclusión, ésta tesis doctoral demuestra que los ácaros *Demodex* son habitantes normales de la piel canina y que se distribuyen en pequeño número a lo largo del pelaje canino. Además, los ácaros *D. canis* deben ser considerados una especie diferente a ácaros *D. injai* y, los ácaros *D. cornei*, una variante morfológica del mismo. Por otro lado, los perros sanos y los perros con demodicosis juvenil generalizada presentan una respuesta humoral adquirida y anticuerpos dirigidos contra diversos antígenos proteicos de *D. canis*.

RESUM

Els àcars *Demodex* són habitants normals de la pell dels mamífers. Els mateixos s'han adaptat a viure dins dels fol·licles pilosos i les glàndules sebàcies de la pell dels seus hostes mamífers. La demodicosis pot ser definida com una malaltia inflamatòria cutània caracteritzada per la presència d'una sobrepoblació d'àcars *Demodex*. Es considera que la resposta immunitària cel·lular és la responsable del control de la població d'àcars, mentre que el paper de les respostes immunitàries humoral i innata, son desconegudes. En els animals domèstics, la forma més greu de la demodicosis la pateixen els gossos. En conseqüència, la demodicosi canina és la malaltia produïda per àcars *Demodex* més estudiada. S'han identificat dues espècies d'àcars *Demodex* en els gossos: *Demodex canis* i *Demodex injai*; mentre s'ha proposat una tercera espècie nomenada *Demodex cornei*. Molts estudis han tractat de documentar la prevalença d'àcars *Demodex* en els gossos sans mitjançant diferents mètodes.

En la present tesi doctoral, descrivim una tècnica de reacció en cadena de la polimerasa (PCR) a temps real per detectar ADN de *Demodex* (seqüència del gen de la quitina sintetasa) en mostres de pèls de gossos. Aquesta tècnica va demostrar la presència d'àcars *Demodex* en percentatges més alts que en els estudis anteriors, suggerint que els àcars *Demodex* estan presents en tots els gossos sans independentment de l'edat, sexe, raça o pelatge. A més, aquesta tècnica va demostrar que les poblacions de *Demodex* estan distribuïdes al llarg del cos dels gos en petit números.

Amb el objectiu d'establir les relacions filogenètiques entre les dues espècies canines i la proposta tercer espècie, van amplificar i seqüenciar un fragment del ADN mitocondrial de la subunitat ribosomal 16S. L'anàlisi filogenètica va demostrar que *D. injai* és una espècie diferent de *D. canis*. A més, va demostrar que *D. cornei* és probablement una variant morfològica de *D. canis*. També, es va desenvolupar i es va estandarditzar una tècnica de PCR convencional per a la detecció específica de ADN de *D. injai*. Aquesta tècnica va demostrar que *D. injai* també és un habitant normal de la pell d'alguns gossos. A més, es va demostrar que en la majoria dels casos clínics de demodicosi canina, la sobrepoblació de *D. injai* és infregüent.

Finalment, per tal d'ampliar els coneixements en la resposta humoral de la demodicosi canina, vam obtenir un extracte antigènic cru de *D. canis* i vam demostrar, mitjançant una tècnica de Western-blot, la presència d'immunoglobulines G enfront diversos antígens de *D. canis* en el sèrum sanguini de gossos sans, de gossos amb demodicosi juvenil generalitzada amb o sense infecció cutània secundària.

En conclusió, aquesta tesi doctoral va demostrar que els àcars *Demodex* són habitants normals de la pell canina i que es distribueixen en petits números al llarg del pelatge caní. A més, els àcars *D. canis* ha de ser considerats una espècie diferent a àcars *D. injai* i, els àcars *D. cornei*, una variant morfològica del mateix. D'altra banda, els gossos sans i els gossos amb demodicosi juvenil generalitzada presenten una resposta humoral adquirida i anticossos dirigits enfront diversos antígens proteics de *D. canis*.

3. INTRODUCTION

3. 1. THE GENUS *DEMODEX* AND SPECIES

Life on earth is scientifically classified by taxonomy in a complex web of hierarchically organized organisms. Mites belong to the largest animal phylum in the animal kingdom, which is known as Arthropoda. Arthropods are characterized morphologically by having bilateral symmetry, their bodies divided into rings or segments, jointed appendages, hard chitinous exoskeleton (cuticle) partitioned into chitinous plates, and body cavity called haemocele. On the other hand, ecdysis or moulting is a phenomenon characteristic of all arthropods whereby the cuticle is shed at regular intervals in order to accommodate the growing tissues. 1 Belonging to the class Arachnida subclass Acari, mites can be found in any ecosystem on earth,2 which make them ubiquitous in nature.³ Co-existence of terrestrial vertebrates and arthropods has been continuous over 200 million years.⁴ Parasitism by mites is widespread and involves all the classes of vertebrates, from fishes to mammals. Owing to their small size and their great plasticity, mites are able to adapt to a wide range of habitats. Most families (such as *Demodicidae*) of the superfamily *Cheyletoidea* comprise permanent parasites of vertebrates. 6 It is presumed from the wide distribution of these mites on birds (Harpirhynchidae) and mammals (Psorergatidae and Demodicidae), that a common ancestor could have occurred on the common ancestor of birds, reptiles, and mammals (amniotes). It is also speculated that during the evolution of mammals, hair might be derived from two different morphogenetic processes: (1) a progressive change in the morphogenesis of scales present in some reptilian ancestors of the Carboniferous-Permian Period or (2) a progressive change in the process of morphogenesis of glands in amphibious synapsids in the Upper Carboniferous period. Amniotes originated on islands in coal swamps 300 million years ago and gave raise to two main lineages: the sauropsids and the therapsids. 10,11 The therapsids evolved into theromorphs in the Triassic and Jurassic, 230–130 million years ago. Hairs and vibrissae were present in some lineages of theromorphs. Differently from sauropsids, the skin of therapsids reduced scaling over most of the body and produced rod-like appendages, the hairs, which gave origin to the pelage. The pelage provided thermoregulatory, sensorial, and mechanical functions, and its appearance was one of the key steps in mammalian evolution.1

The genus *Demodex* belongs to the order *Trombidiformes*, suborder *Prostigmata* and *Demodicidae* family. They have been adapted to live inside skin hair follicles and sebaceous glands of mammalian hosts. ¹² Considering this scenario, it is hypothesized that *Demodex spp.* establishment as a permanent parasite of mammals, had to have arisen at the appearance of the first animals with skin hair follicles. It is unknown when this permanent establishment took place, but it is probable that mites of the family *Demodicidae* were originally pilicolous mites like *Myobia* (modified setae to attach to mammal hair), living at the base of the hairs of the host, and have become internal parasites. The very small size and elongation of the body meant special adaptations for this mode of life. ¹³ In this case, evolution of the genus *Demodex* could have been regressive type, where the host and the parasite followed a parallel course of evolution, although they went in opposite directions. ⁵

Human skin Demodex mites were first seen and mentioned in print by Henle in Zurich in 1841. 13,14 Freiedrich Gmeiner, credits the discovery of this parasite to Berger, a Frenchman who found *Demodex* mites in human ear wax in 1842. 15 However, neither of the initial discoverer's work came to light until after the work of Gustav Simon in 1842¹⁶ that discovered the parasite independently when studying the pathological findings of pimples. He named the mite as Acarus folliculorum, and stated that he found them in the skin follicles of the nose of all human individuals except newborn babies. The genus name "Demodex" was designated in 1843 by the zoologist Richard Owen, borrowing from the Greek the words 'demo' (=lard) and 'dex' (=boring worm) to describe the form and preference location of this organism. Later, in 1859, Franz von Levdig made the same description but named the mite as *Demodex* hominis. Furthermore, he described and named two additional mite species in dogs and Surinam bats: Demodex canis and Demodex phyllostomatis, respectively. 17 Since then, many species of *Demodex* mites have been described. Mites have been retrieved from multiple mammalian hosts. However, not all of the mite species have been named. In Annex (page X), all Demodex mite species known at the present time are depicted. It is important to emphasize that Demodex mites have only been recovered from mammals, and that the presence of these mites is unknown in many mammalian species.¹⁸

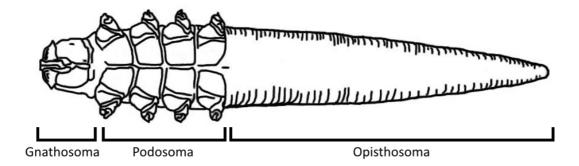
3. 2. THE *DEMODEX* MITE

Despite the existence of multiple *Demodex* species in thirteen different orders of mammalian hosts (*Chiroptera*, *Scadentia*, *Primates*, *Carnivora*, *Pinnipedia*, *Edentata*, *Rodentia*, *Perissodactyla*, *Cetartiodactyla*, *Hyracoidea*, *Lagomorpha* and *Marsupialia*)¹, most studies have focused on the hair follicle mites of man and dogs.

Adaptations to life – the members of the family Demodicidae are one of the most specialized arthropods. This level of specialization has been achieved through evolution of regressive type in which the host and the parasite followed a parallel course of evolution, but in opposite directions.² To survive in such an environment, modifications for life in skin hair follicles, or glands and their outlet ducts, leaded to an extreme reduction in morphological elements.¹

Morphology – the cigar-shaped body is divided into three distinct portions (tagmas): gnathosoma, podosoma, and opisthosoma³ (Figure 1). The gnathosoma has a trapezoidal or rectangular shape, and includes the capitulum or head. On the dorsal side of the gnathosoma there are supracoxal spines of various shapes, and on the ventral side the horseshoe-shaped outline of the pharynx including the subgnathosomal setae. Stubby legs with a pair of forked claws are included in the podosoma. On its ventral side are present the epimeral plates in which behind or partially between them is located the vulva in the case of female mites. The aedeagus (reproductive organ in males) is situated dorsally, in the midline of the podosoma. The opisthosoma is elongated, on its surface are present the characteristic cuticular striations. The opisthosoma may comprise over 80% of the body length.¹ Size and body proportions describe a slight sexual dimorphism. The mouthparts are complex, stiletto-like chelicerae, and gastrointestinal tract is rudimentary and devoid of anus.⁴

Figure 1. Demodex canis, ventral view. Modified from Hirst, 1919.



Life cycle – until optimal *in vitro* culture techniques and conditions allow *Demodex* proliferation in the laboratory, the true life cycle of *Demodex* remains uncertain. Stanley Hirst, in 1919, listed the following life stages for *Demodex* mites: ovum, larva, protonymph, deutonymph, female, and male adults. Later, Sokolovskii suggested that the deutonymphal stage could be omitted when conditions were unfavorable for the mites. Years later, Frank French described the life stages while studying *D. canis* of the domestic dog. He described five stages: ovum, hexapod larva, hexapod protonymph, octopod nymph, and adult. In 1978, Nutting and Desch redescribed *D. canis* assigning in four life stages, which are currently worldwide accepted by the veterinary community: egg, larva, nymph, and adult.

The life cycle was reconstructed by Spickett⁹ in 1961 while studying *Demodex folliculorum*. According to his observations, adults copulate at the opening of the hair follicle. Then, the gravid female enters into the sebaceous gland and lays its eggs. After a period of approximately 60 hours, larva hatches, feeds continuously until molt, and give origin to the protonymph. The later, takes place into the pilo-sebaceous canal. The subsequent molts are more superficial in location, until the deutonymph crawls onto the skin surface. When the deutonymph enters into a hair follicle molts to become adult.

Feeding – all members of the genus *Demodex* pierce cells¹⁰ by projecting two stylets from the preoral opening to puncture the host cells.¹¹ It assumed that *Demodex* mites subsists by feeding on cells (undercutting of epithelium and invasion of glandular acini)^{12,13} and sebaceous products (sebum);¹⁴ although *Demodex* are notoriously resistant to dietary restrictions.¹⁵ When feeding, *Demodex* mites secretes enzymes from two salivary glands for a pre-digestion; then, the liquefied host cell cytoplasm is ingested into the food canal by the action of a pharyngeal pump.¹¹

Environmental changes – all stages of Demodex mites shows negative phototaxy, 9,14,16 which means that migration from one hair follicle to another (nymphs and adults) must be in hours of dim light. Demodex mites are sensitive to changes in temperature, as they stop moving when temperature is lower than 15°C¹⁷ and die in considerable high temperatures. A range between 16-22°C seems the more appropriate temperatures for survival. Changes in humidity are another important factors since Demodex mites are not capable to survive in dry environments. At 14,17

Transmission – evidence of a natural transmission of *Demodex* mites by direct contact has been obtained when studying newborn infants⁴ and pups obtained by cesarean section.¹⁹ Mites are transmitted during later childhood and early adult life by transfer from adult family members.⁴ In dogs, *D. canis* is acquired from the dam during the first few hours of life, probably during suckling.¹⁹

Synhospitality – it is the occurrence of two or more closely related parasite species of the same genus on the one host. ²⁰ Synhospitalic species may have diverged as a result of synhospitalic speciation, or following some modification of life cycles brought about by mutation or by microgeographic

isolation. In the case of the genus *Demodex*, it is common to find two to five synhospitalic parasite species per mammalian host. For example: *Rattus norvegicus*, 5 *Demodex* species; *Canis familiaris*, 2 *Demodex* species, *Bos tauri*, 3 *Demodex* species; *Mesocricetus auratus*, 2 *Demodex* species; *Homo sapiens*, 2 *Demodex* species. In addition to topographic specificity, *Demodex* mites are associated with particular tissues or structural features of the host's skin. Species from different hosts but colonizing the same microhabitat frequently display more similarities than those living on the same host but in different microhabitat. Example for this would include the similarities displayed by *Demodex gatoi*, and *Demodex criceti*. These species inhabits the superficial skin layer (stratum corneum), but are isolated from different hosts: cats and hamsters, respectively. 21,22,23

3. 3. DISEASES OF HUMANS AND DOMESTIC ANIMALS

In mammals, evidence has suggested that *Demodex* mites are transmitted during the first days of life during nursing. As *Demodex* mites are considered permanent parasites of the skin, it is difficult to associate the presence of the mites with certain skin diseases. Many studies have associated demodicosis with different states of debilitation, malnutrition, drugs, and underlying systemic diseases that in one way or another compromise the host immune response. Demodicosis can be defined as an inflammatory skin disease characterized by the presence of an overpopulation of *Demodex spp.* mites in the skin.

Humans are the sole host of two species of *Demodex* mites, namely *Demodex folliculorum* and *Demodex brevis*.⁵ The first one typically resides in the hair follicle nearer the skin surface, whereas *Demodex brevis* is generally found deeper in the sebaceous and meibomian glands.⁶ It is known that the rate of *Demodex folliculorum* in healthy human skin varies between 3% and 50%,⁷ and that this rate increases with advancing age⁸ reaching 100% in humans over the age of 18 years.⁷ However, mite density is normally low (≤5 *Demodex* per cm²) in the adult population.⁹ *Demodex* mites are usually retrieved from the face, particularly from the nose, cheeks, forehead, temples, and chin.¹⁰ An increased number of *D. folliculorum* mites have been associated with dermatosis such as pityriasis folliculorum, rosacea, acne vulgaris, blepharitis, perioral dermatitis, pustular folliculitis, and papulo-pustular lesions of the scalp.^{11,12}

Pityriasis folliculorum is the most frequent demodicosis in humans, with a density of 61 *Demodex* per cm². ¹³ *Demodex* mite overpopulation has also been associated in patients treated with immunosuppressants such as topical glucocorticoids or topical calcineurin inhibitors. ⁸ In children, *Demodex* overpopulation has been connected with leukemia, and infection with the human immunodeficiency virus. ² One study showed an association between HLA Cw2 and HLA Cw4 haplotypes and human demodicosis. ^{14,15} On the other hand, *D. brevis* seems to have a more important role than *D. folliculorum* in the formation of chalazia. ¹⁶

Bovine, equine, and caprine demodicosis are worldwide recognized but uncommon diseases. Bovine demodicosis is associated with an overpopulation of the mite *Demodex bovis*. Although other two species were retrieved from cattle (*Demodex ghanensis*, and *Demodex tauri*), it is not clear if these mites are associated with skin disease. Horses harbor two species of *Demodex* mites: *Demodex caballi* and *Demodex equi*. Equine demodicosis has been associated with chronic long-term glucocorticoid administration. Demodex caprae. Goats of Saanen breed seem to be more predisposed. Demodex caprae.

Demodicosis in cats is considered to be a rare disease. ²¹⁻²⁴ Three mites, *Demodex cati, Demodex gatoi*, and an third unnamed mite species have been described morphologically. *D. cati* is the follicular cat mite. ^{25,26} Demodicosis due to *D. cati* may include lesions such as alopecia, erythema, comedones, scaling, seborrhea, military dermatitis, hyperpigmentation, erosions, ulcers, and crusting, and may have a localized or generalized distribution. ^{24,27} The localized form

involves the eyelids, periocular area, head, or neck. It can also occur as a ceruminous otitis externa. Skin lesions are variably pruritic. Localized demodicosis has been associated with the local administration of inhalant glucocorticoids.²⁴ This form of the disease is usually self-limiting.²⁷ In the generalized form lesions are found on the face and head, neck, trunk, and limbs. Pruritus is variable, but can be intense. Generalized demodicosis due to *D. cati* has been associated with immunosuppression²⁸ and/or systemic diseases such as diabetes mellitus,^{29,30} feline immunodeficiency virus infection,³¹ feline leukemia virus infection,³² hyperadrenocorticism,^{29,33} systemic lupus erythematosus,³² squamous cell carcinoma in situ,^{34,35} chronic upper respiratory tract infection,³⁶ and toxoplasmosis.³² However, some patients with *D. cati* infestation have no apparent underlying disease or history of predisposing drug use.²⁴ Unless the underlying disease can be resolved, demodicosis will probably only be controlled rather than cured.²⁷

D. gatoi inhabit the superficial skin layer.³⁷ These mites are shorter than *D. cati*, and have a broad, blunted opisthosoma.³⁸ Demodicosis due to *D. gatoi* is a pruritic skin disease where dermatitis is often absent (depending on the chronicity and intensity). The major clinical sign is self-induced alopecia due to intense pruritus most commonly affecting the lateral thorax, ventral and lateral abdomen, and medial aspects of the legs.²⁷ Ceruminous otitis externa due to *D. gatoi* has also been reported.³⁹ Concurrent adverse reactions to food, diabetes mellitus and actinic dermatitis have been also reported in cats with demodicosis due to *D. gatoi* ^{40,41} *D. gatoi* is considered to be contagious,⁴⁰ but not all exposed cats will harbor the mites or show any evidence of pruritus.

An unknown third species of *Demodex* in cats has been identified. This mite is longer than *D. gatoi* but shorter than *D. cati.* 42 It was first reported in 1988, 43 and concurrently with *D. cati* infestation and systemic illness in 2005. 21 An outbreak with this mite was subsequently demonstrated in an animal shelter.44 Since then, several cases of third species mite infestation in cats were reported.^{26,42} Clinical signs included variable pruritus, alopecia, erythema, smooth and shiny skin, and thin hair coat. In most of these cases reported, cats had a history of an underlying systemic disease. A molecular study failed to distinct DNA sequences of the third mite species and D. cati, suggesting that the third Demodex species in cat is a morphologically variant of D. cati. 45 However, more recently a novel PCR technique using 16S rRNA gene sequences was able to demonstrate that the third species was in fact different from the two Demodex mites of cats. Sequence of the third species exhibited only 79 and 77% identity with the *D. gatoi* and *D. cati* sequences, respectively.⁴⁶ Recently, a new real-time PCR with three fluorescent TagMan probes was developed and also confirmed that the third feline species was different from D. cati and D. gatoi. 47 This technique claims to distinguish all three feline Demodex mites.

3. 4. CANINE DEMODICOSIS

In domestic animals, the most severe form of demodicosis occurs in dogs. Canine demodicosis (CanD) is the most studied disease produced by *Demodex* mites. Two canine *Demodex* species have been identified: *D. canis* and *D. injai*. A third species was proposed and unofficially named *D. cornei*. Table 1 shows the main features of each canine species, and features of the proposed third species.

Table 1. Comparison of the two canine *Demodex* mite species with features of the proposed third species. ^{2,3,4}

Species	Habitat	Total length	Observations
D. canis	Hair follicle	Male: 192 μm Female: 223 μm	Absence of opistosomal organ in male adult. Opisthosomal organ length of adult female = 10 µm Egg length = 82 µm. Nymph with four pairs of ventral scutes.
D. injai	Sebaceous glands	Male: 367 μm Female: 339 μm	Opisthosomal organ length of female = 20 µm. Egg length = 105 µm. Nymph with three pairs of ventral scutes.
D. cornei (unofficially named)	Epidermis (stratum corneum)	Male: 121 μm Female: 137 μm	Rectangular fourth coxisternal plate, genital pore opens between the fourth coxisternal plate and the opisthosoma, belt-like plate dividing the podosoma and opisthosoma.

3. 4. 1. Clinical presentation of canine demodicosis

D. canis is the hair follicle mite of dogs. Demodicosis produced by *D. canis* is classically differentiated into two main clinical forms: localized demodicosis and generalized demodicosis. There are no uniform criteria in the veterinary literature to differentiate between the localized and the generalized forms. Table 2 summarizes the most commonly accepted clinical criteria to diagnose these two clinical entities.

Table 2. Clinical criteria for classification into localized *versus* generalized demodicosis.

Localized demodicosis	Generalized demodicosis	Reference
	Involvement of an entire body region, more than 5 focal areas and/or paw involvement.	Mueller RS, 2004
	More than 5 localized lesions, involvement of 2 or more feet, or involvement of an entire body region.	Ghubash R, 2006
No more than 4 lesions with a diameter of up to 2,5 cm.		Mueller et al, 2012
Up to 6 skin lesions.	Twelve or more skin lesions.	Miller WH, Griffin CE, Campbell KL. 7 th edition, 2013.

Localized CanD occurs most commonly in young dogs, 1,2 less than a year of age. Lesions are characterized by small to medium sized patches of alopecia, scaling and crusts, comedones, follicular casts, and erythema with variable pruritus. In some cases, secondary superficial pyoderma can be present. These lesions are generally located in the face, head, and forelimbs. In most affected dogs, lesions resolve spontaneously; but some cases could progress to the generalized form. It is unknown if these cases obey to a generalized form from the beginning rather than a truly progression of the localized form of the disease. Bilateral ceruminous otitis externa is considered as another presentation of localized CanD. The localized form rarely recurs.

Generalized CanD usually become apparent in dogs less than 18 months of age. Lesions are similar to localized demodicosis, but they are more numerous and severe (Figure 2). Bacterial secondary skin infections are generally present giving raise to the appearance of papules, pustules, nodules, erosions, and ulcers (folliculitis and furunculosis), which is manifested by intense pruritus and/or pain. When affected dogs reach at this point, CanD potentially life-threatening these disease. ln lymphadenomegaly, lethargy, fever, anorexia, and sepsis may be present.^{4,5} Some cases of generalized demodicosis may be associated with significant interdigital edema, while in others be the only clinical manifestation. When one or more feet are affected, some authors define this presentation as pododemodicosis.^{7,8,9} It is generally accepted that dogs with pododemodicosis carry a poorer prognosis and often require prolonged and alternative therapy to achieve clinical remission.⁵ It is important to note that pododemodicosis must not be considered as an additional form of demodicosis or as a diagnosis per se. It is just a description of the persistent clinical signs localized on the feet region. CanD can also become generalized without the development of secondary pyoderma, ¹⁰ and therefore, disease is less severe.

Figure 2. A Pug dog with juvenile generalized demodicosis.

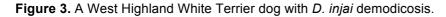


CanD can also be classified according to its onset. Juvenile-onset CanD usually starts during puppyhood (3-18 months), but is not uncommon to make the diagnosis in dogs older than 2 years of age, probably because they went undiagnosed during puppyhood. Adult-onset CanD comprises dogs that have its first episode after 4 years of age. Generally, it is considered that these dogs have an underlying systemic disease that in one way or another compromise the immune system. According to the veterinary literature, multiple conditions were associated with adult-onset CanD: hypothyroidism, hypercortisolism (spontaneous or iatrogenic), leishmaniosis, neoplasia, ehrlichiosis, heartworm disease, and chemotherapy (e.g. cyclosporine, glucocorticoids). However, in more than 50% of cases, no underlying disease is documented at the time demodicosis is diagnosed. Taking into account that most dogs suffering from neoplastic, infectious, or metabolic diseases do not develop demodicosis, diagnose of adult-onset demodicosis in dogs with concurrent systemic disease could be coincidental.

In 1993, it was described a new short-bodied *Demodex* species causing CanD in combination with *D. canis*. This mite resides in the host's stratum corneum, and was proposed as *D. cornei*. In the following years, several reports described additional cases. The majority of these cases were diagnosed as adult-onset generalized demodicosis. So far, there is no sufficient evidence to suggest that cases of CanD with a mixed overpopulation of *D. canis* and *D. cornei* have a poorer prognosis or therapeutic differences.

In the late 1990s, a long-bodied *Demodex* mite, *D. injai*, was identified in dogs. Contrarily to *D. cornei*, which is present in the more superficial layers of the skin, *D. injai* has a deeper ecological niche since it is present in the hair follicles, sebaceous glands, and sebaceous ducts. CanD associated with *D. injai* overpopulation has a striking clinical presentation: most cases reported were associated with a dorsal seborrheic dermatitis (Figure 3). Demodicosis caused by *D. injai* has commonly an adult-onset, and has been associated with different underlying systemic diseases: allergic dermatitis, iatrogenic hypercortisolism, hypothyroidism, and immunomodulatory or

immunosuppressive therapies.^{19,20} According to several reports, terrier dog breeds and their crosses seem to be predisposed to CanD caused by *D. injai*. Histologically, sebaceous gland hyperplasia was associated with this form of CanD, suggesting a correlation between this histological finding, the presence of *D. injai* mites, and clinically evident dorsal greasiness.²⁰





3. 4. 2. Prevalence of canine demodicosis

Prevalence of a certain disease may vary according to many factors such as geographical region, socio-economical factors, covered period of time, and diagnostic criteria. Although CanD is considered a worldwide-distributed skin disease, reports documenting its prevalence are limited. Examples of prevalence of CanD are listed in table 3. Most studies reporting CanD prevalence have been done during a specific period of time (period prevalence), and geographical location. However, results are variable, and difficult to interpret. This is because, among other things, different diagnostic criteria and methods were employed, different source of dogs were included (from stray dogs to veterinary hospital patients), and possibly the most important factor, different conceptual definition of a dog harboring *Demodex* mites *versus* having demodicosis. Most of the studies not include a distinction between localized and generalized demodicosis, or even, juvenile from adult-onset demodicosis. Taking together, results are variable, and difficult to interpret.

Possibly, the most robust and accurate data on canine demodicosis prevalence was reported by Plant et al. in 2011. This retrospective case-control study analyzed clinical records from 750 veterinary hospitals located in 43 states in the United States. Of the 476.635 dogs under 18 months of age, 2.524 were newly diagnosed with juvenile-onset CanD and 243 were identified with pre-existing juvenile-onset CanD.¹ These results provide 2 important data: incidence of juvenile-onset CanD (0,53%) and overall prevalence: 0,58%.

Table 3. Studies reporting CanD prevalence.

Location	Year	Period	Prevalence (%)	Author
Orissa, India	1972	-	67	Mishra SC, et al.
West Bengal, India	1985	-	3.87	Chakrabarti A, et al.
Bendel state, Nigeria	1985	1983	13.29	Ugochukwu El, et al.
United States	1989	1983	0.58	Sischo WM, et al.
Québec, Canada	1990	1987-1988	3.1	Scott DW, et al.
Orissa, India	1997	1987-1992	3.3	Nayak DC, et al.
Taegu, Korea	2000	1997-1998	18.6	Choi W-P, et al.
Uberlândia, Brazil	2003	1994-1998	9.47	Cunha GN, et al.
Mérida, Mexico	2003	2001	23	Rodriguez-Vivas RI, et al.
Aizawl, India	2005	-	35.7	Kalyan S, et al.
Kolkota, and West Bengal, India	2005	2002-2003	10.56	Mahato S, et al.
Assam, India	2005	2000-2001	11.89	Deepa, L, et al.
Gujarat, India	2006	2004	25.45	Solanki JB, et al.
United States	2010	2006	0.58	Plant JD, et al.
Chennai city, India	2011	1998-2006	10.2	Gunaseelan L, et al.
Guangzhou city, China	2012	2009	13.31	Chen Y-Z, et al.
Buenos Aires, Argentina	2013	1998-2006	5.6	Barrientos LS, et al.
Mashhad, Iran	2013	2007-2011	0.94	Khoshnegah J, et al.
Cairo and Giza, Egypt	2013	2010	14.2	Fahmy MM, et al.
Henan, Hebei, Heilongjiang				•
Provinces, and Xinjiang Uygur	2014	2011-2012	25	DonJie C,et al.
Autonomous Region, China				
Dunajpur, Bangladesh	2014	2009-2010	65	Islam MM, et al.

Thus, it is important to distinguish between clinical demodicosis and dogs harboring *Demodex* mites without manifesting demodicosis. Surprisingly, many reports neglected this concept, making the interpretation of results extremely difficult. In table 4, the most relevant studies on *D. canis* prevalence in the skin of healthy dogs are depicted. As shown in table 4, *D. canis* prevalence in canine healthy skin is variable. This can be explained because of the different methods used to confirm the presence of *Demodex* mites, seasonal variations, geographical location, selected cutaneous area, innate resistance, and particularly the age of the animals examined since dogs less than one year of age are more susceptible to *D. canis* infestation probably to constant exposure to the carrier mothers.²

Table 4. Studies reporting *Demodex canis* prevalence in canine healthy skin.

Year	Number of dogs included	Diagnostic method	Demodex canis prevalence (%)	Author
1946	91	Not specified	9.9	Unsworth K.
1958	93	Skin biopsy	5.4	Gaafar SM, et al.
1960	204	Skin biopsy + maceration	52.9	Koutz FR, et al.
1970	120	Skin scrapings	27.5	Avellini G, et al.
1988	89	Skin biopsy	1.1	Henpf-Olchewski C
2003	200	Skin scrapings	3	Rodriguez-Vivas RI, et al.
2006	396	Cotton-wool, combing	1	Ugbomoiko US, et al.
2008	103	Skin scrapings	4.9	Chee J-H, et al.
2010	39	Not specified	43.6	Izdebska JN.
2010	78	Hair plucking	0	Fondati A, et al.
2011	48	Skin scrapings	4.2	Ali MH, et al.
2011	1013	Skin scrapings	7.2	Tsai Y-J, et al.

3. 4. 3. Genetics of canine demodicosis

Generalized demodicosis it is likely to have a hereditary basis. One of the first observations about juvenile CanD was that some dogs seemed to be more susceptible than others, specially the younger ones.² The fact that breeders were able to predict which litters will develop disease³ represented one of the first evidence of a hereditary predisposition to CanD. The first attempts to correlate disease and genetic predisposition included the following factors: age, hair length, developmental stage of sebaceous glands, and skin temperature. Subsequently, experiments performed with anti-lymphocyte serum⁵, and in vitro lymphocyte transformation^{3,6,7} leaded to Scott and others⁸ to hypothesize in 1976 that, canine generalized demodicosis was a manifestation of a specific T-cell defect, probably hereditary in nature. Further research on litters and related dogs suffering from generalized demodicosis showed that the disease was commonly seen in purebred dogs and that a family history could be traced. In 2010, 10 the analysis of microsatellite markers linked to DLA was made in unrelated Boxers, Argentinean Mastiffs and mixed breed dogs suffering from juvenile-onset generalized demodicosis. In this study, a significant association between MHC class II-linked microsatellite alleles (FH2202, FH2975 and FH2054) and the development of demodicosis was demonstrated. This is probably the most convincing published evidence of a genetic background for juvenile-onset generalized demodicosis. In summary, hereditary basis of CanD is based in three pillars: (1) the presentation of the disease in early stages of life, (2) the presentation of disease in litters and related dogs, and (3) the increase risk for disease in certain breeds.

In this manner, knowledge of the hereditary basis of CanD allowed to veterinarians worldwide to establish and improve local breeding programs for breeders. This is particularly demonstrated when certain breeds that in the past were at greatest risk for demodicosis are diagnosed with low frequency in the most recent studies, although results from the past may not be representative because of the lack of statistical analysis. In a recent study, In multivariate analysis of dogs in the United States showed that the following breeds were at high risk for the diagnosis of juvenile-onset generalized demodicosis: American Staffordshire terrier, Staffordshire bull terrier, Chinese shar-pei, French bulldog, Pit bull type, English bulldog, crossbreeds, American bulldog, Boxer, Great dane, Boston terrier, Miniature pinscher, Jack Russell terrier, and Pug.

3. 4. 4. Diagnosis of canine demodicosis

Diagnostic methods to detect *D. canis* on the skin of healthy dogs includes: skin scraping, hair plucking, and skin biopsy. According to table 4, skin scraping is the most commonly used method, and it is considered the standard method for diagnosis demodicosis.¹ Veterinary dermatology textbooks recommendations for practitioners suggest that for diagnosis of CanD, skin scrapes must be properly performed. These include squeezing affected skin to extrude the mites from the hair follicles, and deep and extensive scrapes.^{2,3} Squeezing the skin prior to scraping significantly improves the number of positive samples.⁴ Sensitivity of deep skin scrapings for the diagnose of canine

generalized demodicosis is reported to be of 100%;⁵ although, in one study,¹ positive deep skin scrapes were required as an inclusion criteria. The sensitivity of this technique for the detection of *Demodex* mites in the skin of healthy dogs is unknown.

Hair plucking technique (trichoscopy) was compared with the deep skin scraping technique for the diagnosis of CanD^{1,4,5} although results are inconclusive. Sensitivity of hair plucking technique for the diagnosis of canine generalized demodicosis is reported to be of 97,3%. Hair plucking technique was used for the detection of *D. canis* in the skin of healthy dogs. According to this study, the estimated prevalence of healthy dogs harbouring *D. canis* using hair plucking technique in clinically normal skin does not exceed 5,4%, with 95% confidence level. Hair plucking technique represent some advantages in comparison to deep skin scrapings: it is faster, less harmful, and it can be performed in every haired area of the skin, even in the most difficult to access.

Skin biopsy is the less common performed technique to diagnose CanD because is the most invasive technique, it requires more time, it is more expensive, and it cannot be easily performed in every area of the body. In addition, it is not a suitable technique for diagnosis CanD since only includes small skin proportions to analyze, and the histological sample process may alter the results.⁶

Another two techniques were developed and compared for the diagnosis of CanD. The first one consists in the microscopic analysis of skin exudate. In one study, the relative sensitivity of exudate microscopy was estimated in 100% for diagnosis of generalized CanD. The second technique consists in the microscopic analysis of acetate tape skin impression. Before performing, skin must be squeezed. In one study, acetate tape impression of squeezed skin was found to be as sensitive (100%) as deep skin scraping (90%). The authors claimed that this technique has novel advantages comparing to others, such as, less cell debris during sample observation, which facilitated counting of the mites; less traumatic and painful for the dog; and useful for lesions located in areas that can be particularly difficult to sample such as the paws, interdigital skin, labial commissures and periocular region.

3. 4. 5. Treatment of canine demodicosis

Contrarily to other matters, multiple studies, reviews, and guidelines were published with regard to CanD treatment. Most cases of localized CanD resolve spontaneously in 3 to 8 week, in spite of therapy. However, topical antiseptic therapy may be recommended to prevent or treat a secondary bacterial skin infection. Organophosphorus were one of the first compounds employed for the treatment of generalized CanD6, particularly O, O-dimethyl 0-(2,4,5-trichlorophenyl) phosphorothioate (Ronnel). This compound was associated with several adverse effects, both for the patient and the person applying the treatment. Topical 4 to 8.5% ronnel applied to one third of the body daily to every third day was either used alone or in combination with systemic ronnel at 50-70 mg/kg orally with a success rate of 80 to 100%. Side effects

included salivation, emesis, diarrhea, miosis, bradycardia, muscular tremors, dyspnea, pulmonary edema, convulsions, and death.⁷

Amitraz (N' -(2,4-dimethylphenyl)-N[[(2,4-dimethyl-phenyl)imino]methyl]-N-methyl-methanimidamide) was the first product licensed for use in the treatment of CanD.¹ This emulsion of water and organic solvent is a broad-spectrum miticide that is a member of the formamidine family.² It is applied topically, and previous clipping is thus recommended. Side effects followed therapy with amitraz included: lethargy, depression, ataxia, anorexia, vomiting, diarrhea, polyphagia/polydipsia, hypothermia, pruritus, bradycardia, ataxia, hyperglycemia, and excessive sedation.¹¹² Success rates of amitraz therapy vary between 0% to 100%.¹

The advent of macrocylic lactones supposed a big step forward in the treatment of CanD, mainly because of its safety in mammals and broadspectrum as antiparasitic agents. Avermectins (such as ivermectin, doramectin, selamectin) and milbemycines (milbemycin oxime and moxidectin) families⁸ are the most commonly used macrocyclic lactones for the treatment of CanD. 9-14 Although not licensed for its use in CanD, ivermectin daily oral administration, is an effective therapy for CanD.6 Since the use of ivermectin for CanD treatment, reports have noticed that herding breeds seemed to be more susceptible to ivermectin toxicity. In 2001, a mutation in the ABCB1-1 Δ (formerly MDR1 Δ) gene was described in ivermectin-sensitive Collies. 15 Dogs that are homozygous for the deletion mutation display the ivermectin-sensitive phenotype, while those that are homozygous normal or heterozygous do not display increased sensitivity to ivermectin. In the following years, several other breeds have been identified; 16 however, no all cases of ivermectin toxicosis are explained by an ABCB1-1Δ gene deletion mutation, 17 suggesting that other mechanisms are possible. Signs of ivermectin toxicity are most commonly neurological and include lethargy, tremors, mydriasis, and death. Daily ivermectin therapy for CanD success rate has been referred as 67,5%.

Milbemycin oxime is licensed for the treatment of CanD in some countries. According to several reports, milbemycin, has been studied at two daily oral doses: low dose therapy, and high dose therapy. Although controversially, high dose therapy showed the highest success rate (67%). The success rate of milbemycin oxime was shown to be much lower in dogs with adult-onset CanD. It is also recommended to test for ABCB1-1 Δ gene before milbemycin oxime treatment since adverse effects were detected in dogs homozygous for the deletion mutation; although, in one study 19 17 Collies homozygous for the ABCB1-1 Δ mutation receiving high off-label doses of milbemycin did not showed signs of milbemycin oxime toxicosis. In other studies, the most common clinical signs reported include ataxia, tremors, lethargy, vomiting, mydriasis, disorientation, and hypersalivation.

Moxidectin has been administered for the treatment of CanD as an off-labeled oral, ²⁰ and subcutaneous formulation. ²¹ Success rate is comparable to ivermectin. ⁶ Recent reports, have studied the use of a spot-on formulation. ^{22,23,24,25} Results of one pilot study, ²⁴ suggested that monthly application of a spot-on formulation combining moxidectin and imidacloprid may

be effective as maintenance therapy in relapsing cases of CanD. Adverse effects are similar to those of ivermectin, and they were described more commonly. However, a study performed in P-glycoprotein-deficient CF-1 mice demonstrated that moxidectin had a 2.7-fold lower neurotoxic potential compared to ivermectin. 27

Recently, a novel long-acting systemic insecticide and acaricide, fluralaner, has been proposed as an effective treatment for canine demodicosis.²⁸

3. 5. IMMUNOLOGY OF CANINE DEMODICOSIS

3. 5. 1. The control of *Demodex* mite populations

Evolution of *Demodex* mites followed regressive features to adapt to life in their hosts skin. According to Fain,¹ the progressive regression of the parasite appears to be directly related to the efficiency of the immune system. Many studies have proposed the host immune system as a main controller of *Demodex* mite population.²⁻⁷ This statement is supported by experimental and clinical observations: (1) some dogs and people develop demodicosis when they are treated experimentally (or not) with substances that induce an immunosuppressed state, ^{6,8-10} (2) development of demodicosis in strains of immunodeficient mice, ¹¹⁻¹⁴ and (3) clinical cases of demodicosis induced by immune-deficient diseases, such as leukemia and infection with human immunodeficiency virus in people, ¹⁵ or leishmaniosis, ¹⁶ heartworm disease, hyperadrenocorticism, and ehrlichiosis, among others, in dogs.¹⁷ However, some works in dogs have suggested that immunosuppression occurs during disease and that is not a primary trigger for mite overpopulation.^{18,19} This observation represents one explanation about why not all immunosuppressed dogs develop demodicosis.

Classically, host immune response has been subdivided into an innate and adaptive immune responses, which is branched into a humoral immune response and a cell-mediated immune response.²⁰ Most authors in the veterinary literature have concluded that the main mechanism of control of *Demodex* mite population would be cell-mediated, and that an impaired cell-mediated immunity is present when mite overpopulation overcomes the host immune system.^{6,21-27}

3. 5. 2. Innate immune response in canine demodicosis

Innate immune response, considered the first line of defense, has been poorly investigated in CanD. The innate immune system recognizes molecular structures that are characteristic of microbial pathogens but not of mammalian cells. These structures are called pathogen-associated molecular patterns, and are recognized by host pattern recognition receptor molecules (PRR). Toll-like receptors (TLR) are one of the most important PRR of innate immunity. because they recognize a wide variety of microbial structures. Chitin, after cellulose, is the second most abundant polysaccharide in nature, and is the main component of arthropods exoskeleton. Chitin is found in many body parts of Demodex mites, 2,3,4 and its main function it to protect from environmental conditions and from the host immune responses. One study,⁵ showed that different sized chitin fragments can interact with different innate immune pathways such as TLR2, dectin-1, and NF-kB to stimulate murine macrophage production of IL-17, IL-10, and TNF.⁶ In another study, it was shown that chitin is sensed through TLR2 and induce chemokine release and TLR4 expression in primary human keratinocytes. A study performed on canine keratinocyte cell line CPEK demonstrated that chitin induced a marked increase in the expression of TLR4 and TNF-α.8 Furthermore, an immunohistochemical

controlled study⁹ in skin of dogs with demodicosis showed overexpression of TLR2, which was associated with epidermal hyperplasia and/or spongiosis. This is the only evidence of TLR expression in CanD, although further studies are needed to confirm that *Demodex* mites do stimulate TLR.

3. 5. 3. Cell-mediated immune response in canine demodicosis

Although at first instance CanD was thought to be a delayed type hypersensitivity, most authors in the veterinary literature have concluded that the main mechanism of control of mite population would be cell-mediated, and that an impaired cell-mediated immunity is present when mite overpopulation overcomes the host immune system. 1-8 The first evidence of this arose from experimental studies in puppies treated with azathioprine and anti-lymphocyte serum.^{9,10} In these experiments CanD was spontaneously provoked. Then, many authors focused on canine cell-mediated immunity through in vitro lymphocyte blastogenesis assays, intradermal tests with mitogens, and skin histopathology. In table 5, the most relevant experimental studies conducted to address a cell-mediated deficiency in dogs with CanD are summarized. From these investigations, it is difficult to conclude what are the key immunological defects in spontaneous CanD. Divergence of dogs included (age, breed, health status, concurrent diseases, stage and onset of CanD), absence of a statistical workup, and different inclusion criteria make results of these works inconclusive. and difficult to compare. This was remarked by Barriga in 1992¹¹ when performed lymphocyte blastogenesis assays in dogs with CanD of a same breed and comparable age. He still found non-normally distributed values, and insisted that valid comparisons of lymphocyte blastogenesis results between dogs demand careful statistical analysis. Despite this, these preliminary studies suggest that in CanD a T cell-mediated impairment may be present, and that this impairment resolves when Demodex population is reduced by acaricidal treatment.

 Table 5. Experiments documenting cell-mediated immunodeficiency in CanD.

Author (Year)	Experiment(s)	Finding(s)	Conclusion(s)
Owen (1972)	Neonatal dogs were treated with ALS.	100% developed demodicosis.	Immunosuppression is a key factor for the development of CanD.
Scott et al. (1974)	Serum protein electrophoresis, ID tests with common antigens, and IVLB assay.	Marked elevations of $\alpha 2$ and β globulins, no hyperactivity nor hypoactivity of type I reaction, 32 to 100% of lymphocyte depression, low responses to ID PHA and ConA.	Mites and/or their products may interfere with cell-mediated response.
Hirsh et al. (1975)	PBMC from healthy dogs and dogs with CanD were cultured with autologous/homologous serum and stimulated with PHA.	CanD lymphocytes responded when cultured in PHA and serum from control dogs.	Serum's presence of a lymphocyte suppressive factor.
Corbett et al. (1975)	IVLB: quantification of thymidine uptake with/without PHA.	81% reduction in thymidine uptake whether cultured in autologous/control serum, although serum from dogs with CanD suppressed normal lymphocytes.	Lymphocytes in CanD are suppressed. Serum's presence of a lymphocyte suppressive factor.
Scott et al. (1976)	(1) CanD: IVLB using PHA, ConA, and PWM (2) Healthy dogs: IVLB + mitogens+ CanD serum	(1) Marked lymphocytes unresponsiveness to all mitogens.(2) Healthy lymphocytes were suppressed by CanD serum.	Response to mitogens returned to normal when mite numbers decreased. Evidence of a humoral factor that suppress T-cell function.
Healey et al. (1977)	Weekly blood cell count was performed in control puppies, puppies immunosuppressed with ALS and inoculated with <i>Demodex</i> , and puppies only inoculated with <i>Demodex</i> .	Administration of ALS suppressed the numbers of peripheral blood lymphocytes while mites alone did not. Mean % of lymphocytes was lower in puppies receiving mites than in control puppies.	Cell-mediated immunity is an important line of immune defense against CanD. Mites could have a suppressive effect.
Healey et al. (1977)	ID with PHA and ConA on CanD dogs and SPF dogs.	All have positive reactions, but in CanD dogs reactions subsided at 48 h.	Dogs with CanD have suppressed response to T-lymphocyte mitogens.
Wilkie et al. (1979)	ID tests with PHA in dogs of an inbreeding program from a kennel with high prevalence of CanD.	Cutaneous response was highly depressed in puppies, which were presumed susceptible to CanD.	Impairment of cell- mediated response may exist prior to development of CanD
Krawiec et al. (1980)	IVLB with ConA, and serum globulin fractionation of a litter of puppies whose parents had CanD. Later, puppies also developed CanD.	The 3-week-old puppies lymphocytes had 60% depression of blastogenesis as compared to control adult lymphocytes. As the puppies become older, lymphocytes responded normally to ConA. Sera from newborn puppies, which latter developed CanD, stimulated blastogenesis of control lymphocytes better than control adult serum.	Initial primary lymphocyte unresponsiveness in dogs susceptible to CanD. By the time the lymphocytes are capable of responding, the serum suppressive effects allow the mites to multiply. Blocking factors apparently exist in the serum β fraction of dogs with CanD.

Author (Year)	Experiment(s)	Finding(s)	Conclusion(s)
Barta et al. (1983)	IVLB with ConA, PHA, and PWM. Different serum additions were performed.	There was no detectable presence of blastogenesis suppressing serum factor or lymphocyte dysfunction in the dogs with CanD uncomplicated with secondary pyoderma.	Mites are not the cause of blastogenesis suppression. Serum's presence of a lymphocyte suppressive factor is present only in cases of severe secondary pyoderma.
Kraiß (1987)	IVLB before and after stimulation with muramyldipeptide and PIND-ORF	Muramyldipeptide and PIND- ORF have the effect of raising the lymphocyte response to mitogen in dogs with CanD, however, never reaching the comparative values of healthy controls.	Lymphocytes of dogs with CanD respond to stimulants but not like lymphocytes of healthy dogs.
Barriga et al. (1992)	IVLB with ConA, and PHA, in healthy dogs, dog with GD and dogs with LD, 1 to 3 weeks from the appearance of clinical signs, and 3 weeks later.	1-3 weeks: dogs with GD and LD exhibited responses to mitogens comparable to healthy controls. 6 weeks: dogs with LD exhibited a moderate depression of lymphocyte blastogenesis, dogs with GD showed severe depression to lymphocyte blastogenesis.	Immunosuppression develops with clinical signs of CanD, and is caused by the mites rather than a pre- existing condition.
Caswell et al. (1995)	Histopathologic examination of CanD skin biopsies.	Cells infiltrating the follicular wall and dermal inflammatory infiltrate were predominantly T lymphocytes (CD3+). With the advance of follicular damage, these cells diminished in proportion.	The lesion of interface mural folliculitis represents a cell- mediated immune response targeting the follicular epithelium.
Lemarié et al. (1996)	IVLB with ConA in dogs with GD without pyoderma and control dogs. Quantification of IL2 production and IL2 receptor expression.	Lymphocytes from dogs with GD had a decreased IVLB response compared to controls. Lymphocytes from dogs with GD produced less IL2 and expressed less IL2 receptors than controls.	Dogs with CanD have a decrease cell- mediated response. Serum's lymphocyte suppressive factor could be IL4 or IL10.
Burkett et al. (1996)	IVLB with ConA and CD4+/CD8+ ratio in dogs with juvenile CanD, before and after treatment of pyoderma, and <i>Demodex canis</i> .	Lymphoblastogenesis was suppressed in all CanD dogs with concurrent pyoderma. Lymphocyte response improved with antibiotics alone and with anti-mite treatment, but never approached that of the control, even with eradication of mites. CD4+/CD8+ ratios were higher in CanD dogs than controls before treatment and became normal over time.	Lymphocyte response is suppressed by CanD and concurrent pyoderma. CD4+ or CD8+ lymphocyte subsets may play a role in the initiation or establishment of CanD.
Day (1997)	Histopathologic and immunohistochemical examination of CanD skin biopsies.	CD3+ T lymphocytes were prominent within the interface infilrates of follicular epithelium and also within the lesions of furunculosis.	Dogs with CanD have a local cutaneous cell-mediated immune response.
Caswell et al. (1997)	(1) Histopathologic and immunohistochemical examination of CanD skin biopsies during therapy. (2) Blood flow cytometry.	 (1) Most lymphocytes infiltrating the follicular epithelium in lesions of mural folliculitis were CD3+CD8+. (2) Selective proliferation of CD8+ cells. 	CD8+ cytotoxic lymphocytes are important in the immune response to Demodex canis.

Author (Year)	Experiment(s)	Finding(s)	Conclusion(s)
Toman et al. (1997)	Quantification of cytokine mRNA expression PBMC of dogs with or without CanD, and controls.	Immunosuppression founded in 28.8% of cases was rare in dogs with demodicosis and did not appeared without a concurrent pyoderma.	Deep pyoderma more than demodicosis was concerned with immunosuppression.
Tani et al. (2002)	Quantification of cytokine mRNA expression PBMC of dogs with or without CanD, and controls.	Dogs with CanD had decreased mRA expression of IFNγ and TNFα. mRNA expression of IL5 and TGFβ was higher in dogs with CanD, and decreased with clinical resolution.	Cell-mediated immunity is depressed in CanD.
Fukata et al. (2005)	Quantification of CD4+/CD8+ ratio by flow cytometry of PBL in dogs with juvenile- and adult- onset CanD, and controls. Serum protein electrophoresis.	No differences were observed between the juvenile-onset and the adult-onset groups in the CD4+/CD8+ ratio. In adult-onset CanD the ratio was lower than those in normal and juvenil-onset dogs. Before treatment, values of serum protein, γ globulin, and β globulin were higher in dogs with demodicosis than those in normal dogs. These levels decreased after treatment.	In dogs with CanD showing a low CD4+/CD8+ ratio, the disease is refractory.
Singh et al. (2010)	Quantification of CD4+/CD8+ cells and ratio by flow cytometry in PBMC of dogs with or without CanD, and controls.	Higher numbers of CD8+ T cells and lower numbers of CD4+ T cells were found in dogs with GD compared to healthy dogs and dogs with LD. Higher numbers of CD8+ T cells and lower numbers of CD4+ T cells were also found in dogs with localized demodicosis compared to healthy controls.	CD4+/CD8+ ratio may be involved in the pathogenesis of the generalized CanD.
Singh et al. (2011)	Quantification of apoptotic and dead cells in PBL of dogs with CanD and healthy controls.	Dogs with GD revealed higher apoptotic leukocytes than dogs with LD and healthy controls. Dog with LD showed higher apoptotic leukocytes than healthy controls.	Premature apoptosis of PBL may be implicated in the immunosuppression of the dogs with CanD.
Yarim et al. (2013)	Plasma and serum quantification of PDGF-BB and TGFβ1 by ELISA in dogs with CanD and healthy dogs.	Marked increases in plasma PDGF-BB and in serum TGFβ1 concentrations were detected in dogs with CanD as compared to healthy dogs.	Increased concentrations of circulating PDGF-BB and TGFβ1 play a pivotal role in the pathogenesis of CanD.

ALS, anti-lymphocyte serum; ID, intradermal test; IVLB, in vitro lymphocyte blastogenesis; PHA, phytohemagglutinin; ConA, concavalin A; PWM, pokeweed mitogen; SPF, specific-pathogen-free; LPS, lipopolysaccharide; GD, generalized demodicosis; LD, localized demodicosis; CD, cluster of differentiation; IL, interleukin; PBMC; peripheral blood mononuclear cells; mRNA, messenger ribonucleic acid; INF; interferon; TNF, tumor necrosis factor; TGF, transforming growth factor; ELISA, enzyme-linked immunosorbant assay; PDGF, platelet derived growth factor; PBL, peripheral blood leukocytes.

3. 5. 4. Humoral immune response in canine demodicosis

There are few works addressing this topic in immune response in CanD. This might be a reflection of the absence of known *Demodex* mite antigens. So far, there is no culture media to grow *Demodex* mites; hence, there is no availability of commercial *Demodex* protein extracts to perform assays. Subsequently, the few studies that evaluated the humoral response in CanD had to perform experiments that approached this issue in an indirect way. Table 6 shows the results of these works.

When performing in vitro lymphocyte blastogenesis assays, authors became aware of the presence of an unknown serum factor that suppressed T proliferation. 1-5 This factor was termed serum's lymphocyte immunoregulatory factor (SLIF).6 However, the presence of SLIF could not be demonstrated in dogs with generalized demodicosis without complicating secondary pyoderma. In this way, secondary pyoderma was suggested to play a paramount role in the field of immunosuppression rather than the presence of Demodex overpopulations. 8 As it can be observed in table 6, with the advent of newer technologies, what was previously defined as an unidentified serum factor, recent studies point to circulating cytokines such as IL10/IL4,9 or TGFβ1.^{10,11}

Taking together, newer approaches to enlighten the field of humoral response in CanD are needed, for example, the production of a *Demodex* mite antigen.

 Table 6. Studies addressing the humoral response in CanD.

Author (Year)	Experiment(s)	Result(s)	Conclusion(s)
Hagiwara et al. (1974)	Serum protein electrophoresis.	Dogs with CanD showed reduced albumin and increasing proportions of $\alpha 2$, β or γ according to the extent and age of skin lesions compared to healthy controls. The total serum protein value was not affected.	In generalized CanD there is an increase in the β and γ globulin fractions, which is not present in localized CanD.
Scott et al. (1974)	Serum protein electrophoresis.	Mild to marked elevations of $\alpha 2$ and β globulin fractions.	Non-specific immunosuppressive factors may be present in the serum α2 fraction.
Corbett et al. (1975)	Injection of aleutian mink disease virus.	Both control dogs and CanD dogs responded with quantitatively similar titers.	No humoral response deficiency.
Healey (1977)	Serum radioimmunoelectrophoresis.	Anti-mite Igs in the sera of dogs with CanD or SPF dogs was not detected.	Absence of anti-mite Igs in the sera of dogs with CanD.
Healey et al. (1977)	Detection of IgE bounded to mast cells in skin biopsies of dogs with CanD and SPF dogs.	The percentage of fluorescing mast cells of dogs with CanD and SPF dogs was not different.	Since the number of mast cells in the dermis of dogs with CanD is greatly increased, there may be a higher concentration of IgE in these dogs.
Scott (1979)	 (1) Quantification of antibody titer against canine distemper virus and infection canine hepatitis virus. (2) Quantification of plasma cells in different tissues of dogs with CanD. (3) Serum protein electrophoresis. 	(1) Dogs with CanD developed normal antibody titers after vaccination. (2) Plasma cells are normal or elevated in number (3) Elevations of $\alpha 2$ and β globulin fractions. An elevation in the γ globulin fraction is less consistent, usually accompanied by secondary pyoderma.	No evidence of humoral deficiency. In fact, the B cell response appears to be excessive.
DeBoer et al. (1988)	Detection of circulating immune complex by solid-phase C1q-binding ELISA in dogs with skin disease and healthy dogs.	Dogs with CanD had higher mean circulating immune-complex than healthy dogs.	Presence of circulating immune-complex in dogs with CanD may have pathogenic significance or may be the result of the disease process.
Day et al. (1988)	Quantification of serum levels IgA by single radial immunodiffusion in healthy dogs, and in dogs with chronic diseases.	The variance of serum IgA values was greater in dogs with CanD than healthy controls.	
Hill et al. (1995)	Quantification by ELISA and immunodiffusion of total serum IgE, IgA, and IgG in atopic, parasitized dogs (3/16 had CanD), and healthy controls.	No differences for IgE between the 3 groups. IgA concentrations were lower in atopic and parasitized dogs compared with healthy controls. IgG levels were higher in atopic and parasitized dogs compared to healthy controls.	Importance of IgG in the humoral response.

Author (Year)	Experiment(s)	Result(s)	Conclusion(s)
Caswell et al. (1995)	Histopathologic and immunohistochemical examination of CanD skin biopsies.	35% of the mononuclear inflammatory cells in the perifollicular dermis stained positive for cytoplasmic IgG. IgG-secreting plasma cells formed a rim at the periphery of perifollicular granulomas, where they constituted 50-75% of the cells. 2-23% of the mononuclear cells in the suppurative dermal lesions of furunculosis were IgG positive.	Shift from Th1 toward Th2 responses in cases of furunculosis.
Shearer et al. (1997)	Quantification of IgG, IgA, and specific antistaphylococcal IgG and IgA in CanD dogs with pyoderma, and healthy controls.	Serum IgG, IgA, and specific anti-staphylococcal IgA concentrations in dogs with CanD with pyoderma was comparable to healthy dogs. Dogs with CanD and pyoderma had increased specific antistaphylococcal IgG concentrations than healthy controls.	Importance of IgG in the humoral response.
Hammerberg et al. (1997)	Detection and quantification by ELISA of circulating IgG anti-IgE in Foxhound dogs with CanD, and healthy controls.	Dogs with CanD had increased levels of circulating IgG anti-IgE compared to healthy dogs.	Allergen processing and presentation in the skin, or genetic predisposition to production of IgG specific for heat labile epitopes of IgE may be linked, or not, to predisposition to CanD.
Day (1997)	Histopathologic and immunohistochemical examination of CanD skin biopsies.	Plasma cells were prominent within all types of histological lesions, and plasma cells expressing cytoplasmic IgG (IgG2 and IgG4) were invariably more numerous than those bearing IgM or IgA.	The relative decrease in tissue IgA-bearing plasma cells may indicate a lack of T-cell derived cytokines that drive the IgA "class switch" in differentiation of B lymphocytes in skin and regional lymph nodes.
Caswell et al. (1997)	Histopathologic and immunohistochemical examination of CanD skin biopsies.	CD21+ B lymphocytes were present in the perifollicular dermis but not in the follicular epithelium.	Cells capable to produce Ig are present in the dermis of cutaneous lesions of CanD.
Mozos et al. (1999)	Histopathologic and immunohistochemical examination of CanD and cutaneous leishmaniasis skin biopsies.	Numerous IgG+ plasma cells, fewer IgM+ and IgA+ plasma cells were found in both cutaneous diseases. IgG4+ predominated over IgG2+ and IgG3+ plasma cells. In contrast, dogs with CanD alone, both IgG2+ and IgG4+ plasma cells were numerous in the associated infiltrate.	The local humoral response appears not to be suppressed in CanD, cutaneous leishmaniasis, or combined infections. However, this humoral response alone was unable to prevent progression of the diseases.

Ig, immunoglobulins; ELISA, enzyme-linked immunosorbant assay.

4. OBJECTIVES

GENERAL OBJECTIVES

Although during the last four decades, there has been much progress in the diagnosis of CanD, many fundamental aspects of the parasite and the host-parasite relationship are unknown. In particular, the fact that all dogs harbors *Demodex* mites on their skin, although it has been proposed as a hypothesis and in agreement with data from other species, it has never been demonstrated formally. In this doctoral thesis we have proposed to specifically address the following questions:

- (1) Considering the limited sensitivity of the diagnostic techniques of CanD, is it possible to develop a molecular technique of high sensitivity to perform epidemiologic studies?
- (2) Is Demodex canis a normal inhabitant of the skin of most (if not all) dogs?
- (3) How many different species of *Demodex* mites live in the dog's skin? Particularly, are the mites with reported different morphologies different species or these morphologies are different features of a single species?
- (4) If different species inhabits the canine skin, is it possible to develop a molecular technique that could distinguish between canine *Demodex* species?

In the other hand, the host immune response and the mechanisms of control of *Demodex* population, although its paramount importance, are partially known. In this way, we have proposed to specifically answer:

(5) Does healthy dogs and dogs with demodicosis produce antibodies against the *Demodex* mite?

SPECIFIC OBJECTIVES

STUDY 1

To develop a real-time polymerase chain reaction (PCR) technique to detect *D. canis* DNA on different tissue samples.

STUDY 2

To amplify and sequence a segment of the mitochondrial 16S rDNA from *D. canis* and *D. injai*, as well as from the D. *cornei* and to determine their genetic proximity.

STUDY 3

Determine whether *Demodex canis* mites are normal inhabitants of the skin of dogs or if only a subset of the canine population harbors the mites.

STUDY 4

To develop a PCR technique that specifically detects *D. injai* DNA on different skin and hair samples of healthy dogs and of dogs with demodicosis.

STUDY 5

Produce a *Demodex canis* antigen extract and detect circulating antibodies against *Demodex canis* in the sera of healthy dogs, and in dogs with juvenile generalized CanD (with/without secondary pyoderma).

5. STUDY 1

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Authors:

Ivan Ravera^{*}, Laura Altet[†], Olga Francino[†], Mar Bardagí^{*}, Armand Sánchez[†], and Lluís Ferrer^{*}

- * Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.
- † Servei Veterinari de Genètica Molecular, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.

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Development of a real-time PCR to detect *Demodex canis* DNA in different tissue samples.

Abstract

The present study reports the development of a real-time polymerase chain reaction (PCR) to detect *Demodex canis* DNA on different tissue samples. The technique amplifies a 166-bp of *D. canis* chitin synthase gene (AB 080667) and it has been successfully tested on hairs extracted with their roots and on formalin-fixed paraffin embedded skin biopsies. The real-time PCR amplified on the hairs of all 14 dogs with a firm diagnosis of demodicosis and consistently failed to amplify on negative controls. Eleven of 12 skin biopsies with a morphologic diagnosis of canine demodicosis were also positive. Sampling hairs on two skin points (lateral face and interdigital skin), D. canis DNA was detected on nine of 51 healthy dogs (17.6%) a much higher percentage than previously reported with microscopic studies. Furthermore, it is foreseen that if the number of samples were increased, the percentage of positive dogs would probably also grow. Moreover, in four of the six dogs with demodicosis, the samples taken from non-lesioned skin were positive. This finding, if confirmed in further studies, suggests that demodicosis is a generalized phenomenon in canine skin, due to proliferation of local mite populations, even though macroscopic lesions only appear in certain areas. The real-time PCR technique to detect D. canis DNA described in this work is a useful tool to advance our understanding of canine demodicosis.

Introduction

Canine demodicosis is a severe and highly prevalent dermatitis caused by the proliferation of *Demodex canis* mites in hair follicles and sebaceous glands. D. canis is considered to be a normal inhabitant of canine skin and the disease is thought to be the consequence of a genetically mediated specific immunodeficiency that allows the proliferation of the *Demodex* mites. Destruction of the hair follicles, the subsequent inflammatory reaction and secondary infection with *Staphylococcus pseudointermedius* are the causes of severe lesions in affected dogs. 1,5

Despite the high prevalence and severity of the disease, many aspects of canine demodicosis remain poorly understood. For instance, the fact that all dogs harbor *Demodex* mites in the skin has never been proven in a sound manner. Most textbooks refer to the classic research done by Gafaar et al., in which *Demodex* mites were found in the skin of 5.4% of healthy dogs. The authors concluded that *Demodex* mites could be found on healthy animals and that follicular mange is a complex condition, but not that all dogs harbor mites in their skin. Similarly, in more recent studies, the authors have not been able to find mites on normal dogs. Rodríguez-Vivas et al. could only find *Demodex* mites in skin scrapings in 3% of healthy dogs in Yucatán, Mexico. Fondati et al., using trichoscopy, could not detect *D. canis* mites in any of the 70 dogs examined, and in only one dog did they find a *D. injai* mite. The authors offered

the weak sensitivity of the techniques used in the studies as a possible explanation for the low results.

In this study, we describe a real-time polymerase chain reaction (PCR) to detect *D. canis* DNA on different tissue samples, with the aim of providing a sensitive and specific tool to help in the understanding of this important canine disease.

Materials and methods

Dogs

Fourteen dogs affected by generalized demodicosis were used as positive controls. The animals presented clinical signs characteristic of the disease, and the diagnosis was confirmed by finding different stages of *D. canis* mites in the trichoscopy and in skin scrapings. Two West Highland white terriers with generalized demodicosis caused by *D. injai* and a French bulldog with a generalized demodicosis caused by an unnamed short-bodied Demodex species (unofficially labeled *D. cornei*) were also included in the study.

Fifty-one healthy dogs attended at the Veterinary Teaching Hospital of the Universitat Autònoma de Barcelona for preventive medical care were also included in the study. Ages ranged from 6 months to 12 years, and 18 different breeds were represented.

Hair samples were obtained by doing a continued traction in the direction of the growth of the hair to include the hair root in the sample. In the case of healthy dogs, the hair samples were taken in all cases from the lateral face and from the interdigital skin of the feet. Each sample included between 250 and 300 hairs with their roots. In the case of animals affected by demodicosis, the samples were taken from lesioned areas. In six of the dogs with demodicosis, hair samples were also obtained from the lateral face and from the interdigital skin, although no lesions were observed in these areas.

Biopsies

Twelve formalin-fixed and paraffin-embedded skin biopsies from dogs and one biopsy from a cat in which *Demodex* mites were identified histologically were also investigated using the real-time PCR technique. In all cases, several mites were present and the histological lesions were characteristic of demodicosis. Although identification of the species is not possible in histologic sections, on the basis of the parasite morphology and localization of lesions, in the canine cases the mites were assumed to *D. canis* and in the feline case *D. cati.* Twenty-micron sections were cut, deparaffinised and used for the real-time PCR.

Real-time polymerase chain reaction

DNA from paraffin-embedded skin biopsies was recovered using the same protocol as described elsewhere. Hair samples were conserved in physiological serum and stored at -20°C until DNA extraction. DNA was recovered with the same protocol mentioned above with some modifications. Briefly, samples were centrifuged in a microcentrifuge at maximum speed for 30 min; once the supernatant was removed, 200 µl of digestion buffer (50 mM)

Tris-HCl, pH 8.5; 1 mM EDTA) and 4 µl of proteinase K solution (10 mg/ml) were added, and samples were incubated at 56°C overnight. After inactivation of the proteinase K for 10 min at 95°C, the samples were centrifuged for 10 min at maximum speed. Supernatant was transferred to a new tube and diluted 1/10 for PCR amplification. Real-time PCR was carried out in a final volume of 20 µl using FastStart Universal SYBR Green Master (Roche), 0.3 µM of each primer and 4 µl of diluted DNA. Duplicates were amplified for each sample. Primer follows: canis pairs used were as D. forward GATGAAGCGGCGAGTAATGTTC-3' 5'and D. canis reverse GACTCCATCTTTACGATGTCTGATTT-3'. They amplified a 166-bp fragment of chitin synthase gene.

The eukaryotic 18S RNA Pre-developed TaqMan Assay Reagent (Applied Biosystems, Foster City, CA) was used as internal reference for dog genomic DNA amplification to ensure suitability of each sample for PCR amplification and to be certain that negative results corresponded to true negative samples rather than to a problem with DNA loading, sample degradation, or PCR inhibition. Water was used as a negative control for the PCR. Positive PCR controls were obtained from clinical samples that had been amplified previously and sequenced to confirm *Demodex* (see below).

Thermal cycling profile was 50°C 2 min and 95°C 10 min followed by 40 cycles at 95°C 15 s and 60°C 1 min. PCR specificity assessment was performed by adding a dissociation curve analysis at the end of the run. The product of the real-time PCR was sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) using the same primers and sequences obtained were compared with GenBank database (www.ncbi. nlm.nih.gov/BLAST).

Results

The real-time PCR was positive in all hair samples from the 14 dogs with demodicosis; these had been positive in the previous microscopic examination. In contrast, negative controls gave consistently negative results. The amplified fragment was of 166-bp as expected, and its sequence matched in the gene bank with chitin synthase of *D. canis*, confirming the specificity of the reaction.

The hair samples coming from the two dogs with demodicosis due to *D. injai* and from the dog with the unnamed short-bodied *Demodex* were also positive, confirming that the technique is useful to detect any of the *Demodex* mites of the dog. The sequencing of the amplification product of the unnamed short-bodied *Demodex* was identical to the product of the amplification of *D. canis*. However, the fragment amplified from the two *D. injai* samples showed changes in seven of the 166 nucleotides.

Eleven of the 12 canine biopsies of dogs with demodicosis were also positive, showing that the technique also worked on formalin fixed and paraffinembedded tissue samples. However, the biopsy of a cat with demodicosis was negative.

Forty-two of the 51 healthy dogs were negative in samples from both the interdigital skin and lateral face. In six dogs, one of the two samples was

positive and three dogs both samples were positive. However, in four of the six dogs with demodicosis the samples taken from non-lesioned skin were also positive.

Discussion

This paper describes a sensitive PCR technique that amplifies *D. canis* DNA not only on tissue samples, such as hairs, but also on formalin-fixed paraffin embedded material. The false negative result in one of the canine biopsies was probably a consequence of the poor condition of the sample, which had remained in standard non-buffered formalin for an undetermined period and then for years in paraffin.

Two previous reports have attempted to amplify *Demodex* DNA with only partial success. Toops et al.¹⁰ could not amplify *Demodex* DNA using primers designed from DNA sequences obtained by a random amplified polymorphic DNA (RAPD) approach, nor by using primers designed from the same sequence of chitin synthase from the Genbank that we used (AB 080667). Borgo et al.,¹¹ using a conventional PCR with primers designed from the same Genbank sequence, were partially successful, and an amplification product was obtained in nine of 12 samples containing *D. canis* mites. Both the design of the primers and the use of a real-time PCR could explain the success of our technique in comparison with previous attempts.

Considering that both *D. injai* and the unnamed short-bodied *Demodex* containing samples were amplified using the *D. canis* primers, we can conclude that our technique is useful to detect any of the *Demodex* mites of the dog, and probably other *Demodex* species. Although not available when the primers were designed, recent data incorporated to the Genbank show that the amplified segment is shared by *D. brevis*, one of the species of *Demodex* living in the human skin. Furthermore, we can also conclude that the unnamed short-bodied *Demodex* and *D. canis* share this 166-bp gene fragment and that, contrarily, the amplification product of *D. injai* shows changes in seven nucleotides. Nevertheless, it has to be underlined that our technique was not designed for species identification purposes.

Using the technique reported here, *Demodex* DNA could be detected in nine of 51 (17.6%) of healthy dogs after sampling hairs on only two points of the skin surface. As shown in Table 1, this figure is notably higher than previous results using microscopic examination of hairs, skin scrapings or digested tissue samples, presumably due to the heightened sensitivity of the real-time PCR reported here. Furthermore, it is foreseen that if the number of samples is increased, the percentage of positive dogs would probably also grow. An interesting finding that merits further investigation is the fact that *Demodex* DNA could be detected in non-lesioned skin of four out of six dogs with demodicosis. This finding, if confirmed in further studies, suggest that demodicosis is a generalized phenomenon in canine skin, due to extensive proliferation of local mite populations, even though macroscopic lesions only appear in certain areas.

Many aspects of canine demodicosis remain unknown or only partially understood, beginning with biology of *D. canis*. It is not known whether all dogs harbor *Demodex* mites in their skin, as all human beings seem to host *D. folliculorum* mites. Alternatively, it may be that only a minor percentage of dogs, or a canine subpopulation composed of dogs of certain age or breeds, harbor the parasite in the skin. Similarly, the regions of the canine skin, which constitute the favorite habitat of *D. canis* are unknown at the present time. The real-time PCR technique described here can be a useful tool in large-scale investigation of the biology of *D. canis* and of the pathogenesis of canine demodicosis.

Table 1. Prevalence of *Demodex* mites on healthy dogs as measured by different authors.

Reference	Technique used	Result: number of positive healthy dogs (% positive)
Gaafar et al. (1958) ⁶	Potassium hydroxide digestion of skin samples + microscopic examination	5/93 (5.4%)
Rodríguez-Vivas et al. (2003) ⁷	Examination of skin scrapings of stray dogs	6/200 (3%)
Chee et al. (2008) ¹³	Examination of skin scrapings of stray dogs	5/103 (4%)
Fondati et al. (2009) ⁸	Trichoscopic examination of hairs	0/78 (0%)
Present results	Real-time PCR on hairs	9/51 (17.6%)

Conclusions

The real-time PCR technique to detect *D. canis* DNA described here is a useful tool to advance our understanding of canine demodicosis. The results obtained in healthy dogs demonstrate that *D. canis* is present in a higher proportion of dogs (17.6%) than previously thought.

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6. STUDY 2

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Authors:

Natalia Sastre^{*}, Ivan Ravera[†], Sergio Villanueva[†], Laura Altet^{*}, Mar Bardagí[†], Armand Sánchez^{*}, Olga Francino^{*} and Lluís Ferrer[‡]

- * Servei Veterinari de Genètica Molecular, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.
- † Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.
- ‡ Department of Clinical Sciences, Cummings School of Veterinary Medicine, Tufts University, 01536 North Grafton, MA, USA.

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Phylogenetic relationships in three species of canine *Demodex* mite based on partial sequences of mitochondrial 16S rDNA.

Abstract

The historical classification of *Demodex* mites has been based on their hosts and morphological features. Genome sequencing has proved to be a very effective taxonomic tool in phylogenetic studies and has been applied in the classification of *Demodex*. Mitochondrial 16S rDNA has been demonstrated to be an especially useful marker to establish phylogenetic relationships. The aim of this study was to amplify and sequence a segment of the mitochondrial 16S rDNA from Demodex canis and Demodex injai, as well as from the short-bodied mite called, unofficially, D. cornei and to determine their genetic proximity. Demodex mites were examined microscopically and classified as Demodex folliculorum (one sample), D. canis (four samples), D. injai (two samples) or the short-bodied species *D. cornei* (three samples). DNA was extracted, and a 338bp fragment of the 16S rDNA was amplified and sequenced. The sequences of the four D. canis mites were identical and shared 99.6 and 97.3% identity with two D. canis sequences available at GenBank. The sequences of the D. cornei isolates were identical and showed 97.8, 98.2 and 99.6% identity with the D. canis isolates. The sequences of the two D. iniai isolates were also identical and showed 76.6% identity with the D. canis sequence. In conclusion, D. canis and D. injai are two different species, with a genetic distance of 23.3%. It would seem that the short-bodied *Demodex* mite *D. cornei* is a morphological variant of D. canis.

Introduction

Since Simon first described *Demodex* in 1842, more than 140 *Demodex* species or subspecies have been identified, infesting hair follicles, sebaceous glands, Meibomian glands and ceruminous glands of numerous mammals, including the dog, horse, sheep, cat, pig and hamster.²⁻⁴ Two or more *Demodex* species might simultaneously parasitize the same mammalian host; this is the case in humans with *Demodex folliculorum* and *Demodex brevis*,⁵ the horse with Demodex caballi and Demodex equi⁶ and the cat with Demodex gatoi. Demodex cati and a third, unnamed species. 7,8 In most mammals Demodex behaves as an opportunistic pathogen with the potential to change its status from commensal to parasite (the mites benefit but harm the host) if the host's cutaneous environment facilitates their proliferation.^{2,9} In this context, the dog seems to be different. It has been demonstrated that all dogs harbour a small number of Demodex mites on the skin, without developing lesions or clinical signs, as occurs in many other mammals. 10,11 However, the dog seems to be special because of the high prevalence of a generalized and severe demodicosis in young individuals, especially in certain breeds, without a known primary or predisposing cause for the mite overgrowth. 12-14 A hereditary defect in the mechanisms of control of Demodex populations is suspected to be the primary cause of canine juvenile demodicosis. 1,2,13,15

Understanding the aetiopathogenesis of canine demodicosis therefore becomes important, not only for improving the control and prophylaxis of one the most serious canine cutaneous diseases, but also for better knowledge of the host–*Demodex* relationship. *Demodex canis* was considered for a long time to be the sole *Demodex* species in the dog until, in the late 1990s, several authors described a mite characterized by a long opisthosoma. This species was morphologically characterized and named *Demodex injai* by Desch and Hillier. Subsequently, several authors described dermatological conditions, mainly seborrhoeic dermatitis, associated with *D. injai* overgrowth, although a causal relationship, as in many forms of demodicosis, was difficult to establish. Interestingly, in cases of generalized juvenile demodicosis with *D. canis* an associated overgrowth of *D. injai* has not been reported to date; presumably, the suspected genetic defect in the control of Demodex populations may be specific for *D. canis*.

The situation became more complex when a third, short-bodied mite, which was named provisionally and unofficially *Demodex cornei*, was described by some authors. Furthermore, some parasitologists have suggested that these are not different species but merely morphological variants of the same parasite. According to these authors, the morphological differences could be the result of circumstances such as the location on the skin surface or the habitat occupied by the mite. ²³

Historically, the speciation of *Demodex* mites has been based mainly on their hosts and morphological features.³ Genome sequencing has proved to be a very effective taxonomic tool in phylogenetic studies and has been applied to the classification of *Demodex* mites. Initially, research groups reported different methods to extract and amplify *Demodex* DNA.^{24,25} Then, different regions of the *Demodex* genome were sequenced to infer phylogenetic relationships between *Demodex* species.^{26,27} Among all genomic regions investigated, mitochondrial 16S rDNA seemed to be an especially useful marker to establish a phylogenetic relationship among closely related species and subspecies, although not for more distantly related taxa.^{28,29} Zhao and Wu,²⁹ amplifying and sequencing this region, were able to identify *D. folliculorum*, *D. brevis* and *D. canis* as different species and to construct a phylogenetic tree of these three *Demodex* species.

The objective of the present research was to amplify and sequence a segment of the mitochondrial 16S rDNA from the two canine *Demodex* species, *D. canis* and *D. injai*, and from the short-bodied *Demodex* mite in order to determine their genetic proximity and to establish their phylogenetic relationships.

Materials and methods

Demodex mites

Demodex mites were isolated individually from skin scrapings from dogs with demodicosis (*D. canis*, *D. injai* and short-bodied *Demodex* mite) or from scrapings of the healthy facial skin of one of the authors (*D. folliculorum*; L.F.).

The sources of the 10 *Demodex* isolates are given in Table 1. The mites were examined microscopically and classified as *D. folliculorum* (one sample), *D. canis* (four samples), *D. injai* (two samples) or the short-bodied *Demodex* species (three samples). The mites were aspirated using a glass micropipette, added to 100 μ L of sterile saline solution and maintained frozen at -20°C. For comparison, the sequences of seven *D. folliculorum*, three *D. brevis* and two *D. canis* mites obtained from Genbank were used.²⁹

Table 1. Sources of the 10 *Demodex* isolates sequenced in the study.

Isolate no.	Source	Morphological identification
1	Facial skin of one of the authors	D. folliculorum
2	Skin of a 10-year-old male poodle with generalized demodicosis	D. canis
3	Skin of a 2-year-old female German shepherd dog with generalized demodicosis	D. canis
4	Skin of a 6-month-old male boxer with generalized demodicosis	D. canis
5	Skin of a 9-month-old French bulldog with generalized demodicosis	D. canis
6	Skin of a 3-year-old West Highland white terrier with seborrhoea	D. injai
7	Skin of a 4-year-old fox terrier with seborrhoea	D. injai
8	Skin of a 7-month-old mixed breed dog with generalized demodicosis*	D. cornei
9	Skin of a 4-month-old Labrador retriever with localized demodicosis	D. cornei
10	Skin of a 6-year-old female mixed breed dog with generalized demodicosis	D. cornei

^{*} The skin scrapings from this dog revealed the presence of *D. canis* mites and also of the short-bodied mites (*D. cornei*); however, for the sequencing only short-bodied mites (*D. cornei*) were selected.

Extraction of DNA

After thawing, samples were centrifuged for 10 min, and the resulting pellet was resuspended in 200 μ L of digestion buffer (50 mmol/L Tris–HCl, pH 8.5; 1 mmol/L EDTA), and 4 μ L of proteinase K solution (10 mg/mL; Roche Applied Science, Sant Cugat, Spain) was added to the samples, which were incubated at 56°C overnight. After inactivation of the proteinase K for 10 min at 95°C, the samples were centrifuged for 15 min at 16,100g. Supernatant was transferred to a new tube for PCR amplification and 1 μ L was used for the PCR.

PCR to amplify 16S mitochondrial rDNA, sequencing and alignment

Similar primers to those described by Zhao and Wu²⁹ were used to amplify a 338-bp DNA fragment of the mitochondrial 16S rDNA gene, as follows: 16S forward, 5'-GTATTTTGACTGTGCTAAGGYAGC-3'; and 16S reverse, 5'-CAAAAGCCAACATCGAGG-3'. Negative PCR controls were used throughout the study in order to detect exogenous DNA contamination. All PCRs were prepared under a laminar flow hood. The DNA from samples was amplified in a PCR mixture containing 1 µmol/L of DNA solution, PCR buffer (1x), 1.5 mmol/L MgCl₂, 0.2 mmol/L of each dNTP, 0.5 µmol/L of each primer and 1 U AmpliTag Gold DNA Polymerase (all from Applied Biosystems, Foster City, CA, USA). All amplifications were carried out in 20 µL reaction volumes. The thermal cycling profile included 10 min at 94°C, followed by 40 cycles of 94°C (30 s), 57°C (30 s) and 72°C (30 s), and then completed with 10 min at 72°C. The PCR products were sequenced with BIG DyeTM Terminator Cycle Sequencing Ready Reaction Kit, version 3.1 (Applied Biosystems), following the manufacturer's protocol. Sequences were purified using the Montage SEQ96 Sequencing Reaction Cleanup Kit (Millipore, Billerica, MA, USA) and analyzed on an ABI PRISM 3730 automated sequencer (Applied Biosystems) in accordance with the protocol provided by the manufacturer. All sequences were examined with SEQSCAPE 2.1.1 (Applied Biosystems), aligned with Bioedit Sequence Alignment Editor, 30 and compared with the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST).

Genetic and phylogenetic analysis

Genetic diversity statistics for 16S rDNA partial sequences, such as the number of haplotypes (H), haplotype diversity (Hd) and nucleotide diversity (π), as well as the nearest-neighbour statistic (S_{nn}), ³¹ were estimated using DNASP 5.10. ³² The significance was determined with the permutation test with 1000 replicates. The nucleotide composition, pairwise genetic identity and P genetic distance of the sequences among 11 *Demodex* specimens were calculated with MEGA 4.0. ³³

Phylogenetic analysis was carried out using 228-bp of the 16S rDNA sequence in *Demodex* and two out-groups (*Aceria guerreronis*-1-DQ063572 and *Tetranychus urticae*-EU345430) in order to root the tree. The best evolutionary model was performed by MODELTEST 3.7³⁴ and selected with Akaike information criterion among 56 models of evolution. Phylogenetic relationships among haplotypes were analysed in PAUP 4.0b10,³⁵ with heuristic searches using 'tree-bisection-reconnection' (TBR) branch swapping under the optimality criteria of maximum parsimony (MP; stepwise addition). Bootstrap analysis³⁶ was run to test robustness of observed branching patterns with 1000 random repetitions for MP. TreeView program³⁷ was used to display phylogenies.

Results

Sequence analysis

The mitochondrial 16S rDNA fragment of all 10 *Demodex* samples was successfully amplified and sequenced. Three new sequences have been submitted to GenBank (accession numbers JX390978, JX390979 and

JX390980). Figure 1 presents the alignments of the 16S rDNA fragments of the 10 *Demodex* isolates and of 12 *Demodex* sequences retrieved from GenBank. For the comparison and phylogenetic studies, a fragment of 228-bp that included the region of highest variability was used. Ten different haplotypes (H) were identified, considering both our 10 specimens and the 12 retrieved from Genbank.

Haplotype (Hd) and nucleotide (π) diversity (excluding gaps) were larger in *D. canis* (n = 9 sequences; H, 4; Hd, 0.750; and π , 0.014) than in *D. folliculorum* (n = 8 sequences; H, 4; Hd, 0.643; and π , 0.011). Genetic differentiation between populations was highly significant (S_{nn} = 1; P < 0.001) due to the fact that they did not share haplotypes. In the same way, the phylogenetic tree (Figure 2) shows that both species were clearly separate (bootstrap value > 95%) in two clades. For *D. injai* (n = 2 sequences) and *D. brevis* (n = 3 sequences), haplotype and nucleotide diversity were not estimated because H = 1 in both cases.

Figure 1. Alignment of the mitochondrial 16S rDNA fragments of the 10 *Demodex* isolates and of 12 *Demodex* sequences retrieved from GenBank. The isolates with the suffix 'UAB' are those sequenced in the present study.

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D. folliculorum-1	1947—AGG COTTITAL A ARTIGGOTT NAGGART TGATTATT AGGARTTATA AGGARTTATA TAGGARTTATA TAGGARTATA TAGARTATA TAGGARTTATA TAGGARTATA TAGGARTA TAGGARTA TAGGARTA TAGGARTA TAGGARTA TAGGARTA TAGGARTA TAGA	
D. folliculorum-2	TARIAUGU GUTTIRI-G ARTUGACUT RAAGTGAATT TGATTITUT RAGAARAGIT AGARGTITU UTARAGACGA GAAGACUCCA ARATUTIRI TITAAT-TIR TAGGATTIA.	LA
D. folliculorum-3		
D. folliculorum-4		
D. folliculorum-5		
D. folliculorum-6	T	
D. folliculorum-7	T	•
D. folliculorum-UAB	T	
D. brevis-1	TT TG AA AT T TTTA G TTT TA GT .GC TA .G. T TCA-GA .GG .ATAG .	
D. brevis-2	TT.TG AA ATT TTTA.G TTT TA GT GC TA G T. T. CA-GA GG ATAG.	
D. brevis-3	TT.TG. AA ATT TTTA.G TTT TA GT GC TA G. T. T. CA-GA GG ATAG.	
D. canis-1	.TGGT. TTACGTTTTGGC AACTTGTTC	
D. canis-2	.TGGT. TTACG. TTT. T. GC AGTCT. GT. TCGAA.AGT	
D. canis-UAB1	.TGG T TTACG .TTT. TGGC AAAAGTTTTGT TC	
D. canis-UAB2	.TGGT TTA .CG.TTT. TGGCACTT.GT TCG	
D. canis-UAB3	.TGGT TTACG.TTT. TGGCACTT.GT TC	
D. canis-UAB4	.TGG T TTACG .TTT. TGGC AAAAGTTTTGT TC	
D. canis (cornei)-UAB1	.TGGT TTACG.TTT. T.GCAGTTT.GT TC	
D. canis (cornei)-UAB2	.TGGT TTACG.TTT. T.GCA GTTT.GT TC	
D. canis (cornei)-UAB3	.TGGT TTACG.TTT. T.GCAGTTT.GT TC	
D. injai-UAB1	AGA.GG.TCG.TTTGATTTTT TC	
D. injai-UAB2	AGA.GG.TCG.TTTGATTT. TCT TC	
	130 140 150 160 170 180 190 200 210 220 230	
D. folliculorum-1		
D. folliculorum-1	TTTTTATTG GGGGAAAGGT TAATTTTAT TTATTGT-T TATTATTGT GAACTTTTTT AGGGTGTATG G-ATAGATAC TTTGGGGGTA ACAGGATTAT TTTCTTT	
D. folliculorum-1 D. folliculorum-2 D. folliculorum-3		
D. folliculorum-2	TTTTATTTG GGGGAAAGGT TAATTTTTAT TTATTGT-T TTATTATTGT GAACTTTTT AGGGTGTATG G-ATAGATAC TTTGGGGGTA ACAGGATTAT TTTCTTTT A. G	
D. folliculorum-2 D. folliculorum-3	TTTTTATTG GGGGAAAGGT TAATTTTAT TTATTGT—T TTATTATTGT GAACTTTTTT AGGGTGTATG G-ATAGATAC TTTGGGGGTA ACAGGATTAT TTCTTTT A. G. — C A. G. — C	
D. folliculorum-2 D. folliculorum-3 D. folliculorum-4	TTTTATTG GGGGAAGGT TAATTTTAT TTATTGT-T TAATTATTG GAACTTTTT AGGGTGTATG G-ATAGATAC TTTGGGGGTA ACAGGATAT TTCTTTT A. G C A. G C	
D. folliculorum-2 D. folliculorum-3 D. folliculorum-4 D. folliculorum-5	TTTTTATTG GGGGAAAGGT TAATTTTAT TTATTGT-T TTATTATTGT GAACTTTTTT AGGGTGTATG G-ATAGATAC TTTGGGGGTA ACAGGATTAT TTTCTTTT A. G C A. G C A. G C	
D. folliculorum-2 D. folliculorum-3 D. folliculorum-4 D. folliculorum-5 D. folliculorum-6	TTTTTATTG GGGGAAAGGT TAATTTTAT TTATTGT—T TTATTATTGT GAACTTTTT AGGGTGTATG G—ATAGATAC TTTGGGGGTA ACAGGATAT TTCTTTT A. G. ——————————————————————————————————	
D. folliculorum-2 D. folliculorum-3 D. folliculorum-4 D. folliculorum-5 D. folliculorum-6 D. folliculorum-7	TTTTTATTG GGGAAAGT TAATTTTAT TTATTGT TTATTATTGT GAACTTTTT AGGGTGTATG G-ATAGATAC TTTGGGGGTA ACAGGATTAT TTTCTTTT A. G C.	
D. folliculorum-2 D. folliculorum-3 D. folliculorum-4 D. folliculorum-5 D. folliculorum-6 D. folliculorum-7 D. folliculorum-UAB	TTTTTATTG GGGGAAAGGT TAATTTTAT TTATTGT—T TTATTATTGT GAACTTTTT AGGGTGTATG G—ATAGATAC TTTGGGGGTA ACAGGATAT TTCTTTT A. G. ——————————————————————————————————	
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D. folliculorum-2 D. folliculorum-3 D. folliculorum-3 D. folliculorum-4 D. folliculorum-5 D. folliculorum-6 D. folliculorum-7 D. folliculorum-7 D. folliculorum-UAB D. brevis-1 D. brevis-2 D. canis-1 D. canis-1 D. canis-2	TTTTTATTG GGGGAAGGT TAATTTTAT TTATTGT TTATTATTG GAACTTTTT AGGGTGTATG G-ATAGATAC TTTGGGGGTA ACAGGATTAT TTTCTTTT A. G C. C. C. T. A. A. A. A. G. GA. A. A. A. G. GA. A. A. A. G. GA. A. C. G. T. G. A. A. A. A. G. GA. A. C. G. T. G. A. A. A. A. G. GA. A. C. G. T. G. A. A. A. A. G. GA. G. A. G. A. T. T. G. G. T. A. A. A. G. GA. G. A. T. T. G. G. T. A. A. A. G. GA. G. A. T. T. T. G. G. T. A. A. A. G. GA. G. T. T. A. A. G. GA. T. T. T. G. G. T. A. A. A. G. GA. G. T. T. T. G. G. T. T. A. A. G. GA. G. T. T. T. G. G. T. T. A. A. A. G. GA. G. T. T. T. G. G. T. T. A. A. C. C. T. A. A. A. G. GA. A. G. A. G. T. T. T. G. G. T. T. A. A. C. C. T. A. A. A. G. GA. A. G. GA. T. T. T. T. G. G. T. T. A. A. C. C. T. A. A. A. G. GA. T. T. T. G. G. T. T. A. A. C. C. T. A. A. A. G. GA. T. T. T. G. G. T. T. A. A. C. C. T. A. A. A. G. GA. T. T. T. G. G. T. T. A. A. C. C. T. A. A. A. G. GA. T. T. T. G. G. T. T. T. G. G. T. T. T. G. C. T. T. A. A. A. G. GA. T. T. T. G. G. T. T. T. G. G. T. T. T. G. C. T. T. A. A. A. G. GA. T. T. T. G. G. T. T. T. G. G. T. T. T. G. C. T. T. A. A. A. G. GA. T. T. T. G. G. T. T. T. G. G. T. T. T. G. C. T. T. A. A. C. C. T. A. A. A. G. GA. T. T. T. G. G. T. T. T. G. G. T. T. T. G. C. T. T. A. A. C. C. T. A. A. A. G. GA. T. T. T. G. G. T. T. T. G. G. T. T. T. G. C. T. T. A. A. C. C. T. A. A. A. G. GA. T. T. T. G. G. T. T. T. G. G. T. T. T. G. G. T. T. T. G. C. T. T. A. A. T. T. T. G. G. T. T. T. G. G. T. T. T. G. C. T. T. A. T. T. G. G. T. T. T. G. G. T. T. T. T. G. T. T. T. T. T.	
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D. folliculorum-2 D. folliculorum-3 D. folliculorum-3 D. folliculorum-4 D. folliculorum-5 D. folliculorum-6 D. folliculorum-7 D. folliculorum-7 D. folliculorum-0AB D. brevis-1 D. brevis-2 D. canis-1 D. canis-2	TITITATE GGGGAAGG TAATTITAT TTATEGT—T TTATTATE GAACTITIT AGGGTGTATG G-ATAGATAC TITGGGGGTA ACAGGATTAT TTCTTTT A. G	
D. folliculorum-2 D. folliculorum-3 D. folliculorum-3 D. folliculorum-4 D. folliculorum-6 D. folliculorum-6 D. folliculorum-0 D. folliculorum-UAB D. brevis-2 D. brevis-3 D. canis-1 D. canis-1 D. canis-UAB1 D. canis-UAB2 D. canis-UAB3	TTTTATTG GGGAAAGGT TAATTTTAT TTATTGT—T TTATTATTG GACTTTTT AGGGTGTAT G—ATAGATAC TTTGGGGGTA ACAGGATAT TTTCTTT A. G. —— C C —— —— —— —— —— —— —— —— —— —— ——	
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D. folliculorum-2 D. folliculorum-3 D. folliculorum-3 D. folliculorum-4 D. folliculorum-5 D. folliculorum-6 D. folliculorum-7 D. folliculorum-UAB D. brevis-2 D. brevis-3 D. canis-1 D. canis-1 D. canis-UAB1 D. canis-UAB2 D. canis-UAB3 D. canis-UAB3 D. canis-UAB4	TTTTATTG GGGAAAGT TAATTTTAT TTATTGT—T TATTATTGT GACTTTTT AGGGTGTAT G—ATAGATAC TTTGGGGGTA ACAGGATAT TTTCTTTT A. G. —— C C —— —— —— —— —— —— —— —— —— —— ——	
D. folliculorum-2 D. folliculorum-3 D. folliculorum-3 D. folliculorum-4 D. folliculorum-5 D. folliculorum-5 D. folliculorum-7 D. folliculorum-7 D. folliculorum-0B D. brevis-2 D. brevis-3 D. canis-1 D. canis-2 D. canis-UABB1 D. canis-UABB2 D. canis-UABB4 D. canis (cornei)-UABB1 D. canis (cornei)-UABB1 D. canis (cornei)-UABB1 D. canis (cornei)-UABB1	TTTTTATTG GGGGAAGGT TAATTTTAT TTATTGT TTATTATTG GAACTTTTT AGGGTGTATG G-ATAGATAC TTTGGGGGTA ACAGGATTAT TTTCTTTT A. G	
D. folliculorum-2 D. folliculorum-3 D. folliculorum-3 D. folliculorum-4 D. folliculorum-5 D. folliculorum-5 D. folliculorum-7 D. folliculorum-WAB D. brevis-2 D. brevis-3 D. canis-1 D. canis-1 D. canis-UAB1 D. canis-UAB3 D. canis-UAB3 D. canis-UAB4 D. canis (cornei)-UAB1	TTTTATTG GGGAAAGGT TAATTTTAT TTATTGT—T TTATTATTG GACTTTTT AGGGTGTAT G—ATAGATAC TTTGGGGGTA ACAGGATTAT TTTCTTT A. G. —— C. C. ——	
D. folliculorum-2 D. folliculorum-3 D. folliculorum-3 D. folliculorum-4 D. folliculorum-5 D. folliculorum-5 D. folliculorum-7 D. folliculorum-7 D. folliculorum-0B D. brevis-2 D. brevis-3 D. canis-1 D. canis-2 D. canis-UABB1 D. canis-UABB2 D. canis-UABB4 D. canis (cornei)-UABB1 D. canis (cornei)-UABB1 D. canis (cornei)-UABB1 D. canis (cornei)-UABB1	TTTTTATTG GGGGAAGGT TAATTTTAT TTATTGT TTATTATTG GAACTTTTT AGGGTGTATG G-ATAGATAC TTTGGGGGTA ACAGGATTAT TTTCTTTT A. G	

Divergence

Table 2 shows the pairwise identity of the specimens sequenced in the present study compared with the sequences registered in GenBank. The sequence of the *D. folliculorum* fragment was identical to the sequence of four Chinese *D. folliculorum* isolates previously published²⁹ and registered with GenBank (accession numbers JF783995, JF83996, FN42425 and FN42426), which in Table 1 appears as *D. folliculorum* sequence 3.

The sequences of the four *D. canis* mites were identical and presented 99.6 and 97.3% identity with the two *D. canis* sequences available from GenBank (accession numbers JF84000 and JF84001), which, according to the authors, correspond to two isolates from a Tibetan Mastiff from China with demodicosis.²⁹ The mean genetic *P* distance inside the species was 1.6%. The sequences of *D. folliculorum* and *D. canis* showed 80.1% identity in our study.

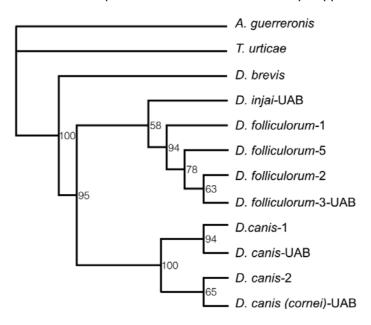
The sequences of the three short-bodied *Demodex* mite isolates were identical and also showed 97.8% identity with the *D. canis* isolates and 98.2 and 99.6% identity with the two sequences of *D. canis* registered with GenBank. The sequences of the two *D. injai* isolates were also identical and showed 76.6% identity with the *D. canis* sequence and 81.9% identity with the *D. folliculorum* sequence (mean interspecific *P* distances of 23.3 and 17.9%, respectively).

Table 3 shows the percentage contents of A, C, T and G. As expected in *Rhinonyssidae* mites, the A + T nucleotide frequencies are considerably higher than G + C frequencies.²⁸

Phylogenetic tree

The best evolutionary model selected by Akaike information criterion among 56 models of evolution was TIM + I + G (that includes invariable sites and the rate variation among sites). Figure 2 shows the phylogenetic tree of the *Demodex* species. In short, *D. brevis* constitutes a separate branch, and the other clade is composed of three branches, one with all *D. folliculorum* sequences, one with *D. injai* and the third with all *D. canis* sequences and those of the short-bodied *Demodex* mite, which are very similar.

Figure 2. Phylogenetic tree of *Demodex* species inferred from partial sequences of mitochondrial 16S rDNA. The tree was constructed as described in the 'Materials and methods' section. Numbers above the lines represent the full heuristic bootstrap support.



D. folliculorum-1 D. folliculorum-2 D. folliculorum-3 D. folliculorum-5 D. folliculorum-UAB D. brevis D. canis-1 D. canis-2 D. canis-UAB Demodex mite-UAB D. injai-UAB 83.3 81.4 81.9 81.9 81.9 81.9 66.2 9.92 77 Table 2. Pairwise sequence identity (as percentages) among four Demodex isolates from the present study ('UAB', Universitat Autonoma de Barcelona) and seven GenBank sequences. Short-bodied 80.5 70.9 98.2 9.66 79.2 80.1 80.1 97.3 81 77 9.62 80.1 78.3 79.2 79.2 70 9.66 97.3 97.8 9.92 79.2 80.1 80.5 80.1 70.9 97.8 97.3 9.66 81 77 80.5 9.62 9.62 78.7 80.1 97.8 98.2 70 77 73.3 71.5 72.9 72.9 72.9 70 70.9 70 70.9 66.2 98.2 98.6 98.6 72.9 9.62 81.9 100 80.1 80.1 98.6 98.6 72.9 80.5 81.9 97.7 97.3 80.1 80.5 98.2 98.6 98.6 72.9 9.62 80.1 80.1 81.9 100 98.6 8.96 98.6 97.3 71.5 81.4 78.7 79.2 78.3 79.2 8.96 98.2 97.7 98.2 73.3 80.5 83.3 80.1 81 8 D. folliculorum-UAB D. folliculorum-5 D. folliculorum-2 D. folliculorum-3 Short-bodied Demodex mite-D. folliculorum-1 D. canis-UAB D. injai-UAB D. canis-2 D. canis-1 D. brevis

Table 3. Nucleotide percentage in *Demodex* specimens.

Demodex haplotype	Т	С	Α	G	Total analysed
D. folliculorum-1	43.9	7.2	29.4	19.5	221
D. folliculorum-2	43	7.7	29	20.4	221
D. folliculorum-3	43.9	7.2	29.4	19.5	221
D. folliculorum-5	43.4	7.7	29.4	19.5	221
D. folliculorum-UAB	43.9	7.2	29.4	19.5	221
D. brevis	42	5.3	32.7	19.9	226
D. canis-1	44.6	7.6	27.7	20.1	224
D. canis-2	45.1	7.1	27.7	20.1	224
D. canis-UAB	44.2	7.6	28.1	20.1	224
D. canis (cornei)-UAB	45.5	6.7	27.7	20.1	224
D. injai-UAB	41.7	7.2	29.6	21.5	223

Specimens with the suffix 'UAB' are those from the present study. The rest of the sequences were retrieved form GenBank.

Discussion

This study confirms that mitochondrial 16S rDNA partial sequence is a useful tool to discriminate between *Demodex* species, as previously reported by Zhao and Wu.²⁹ Demodex canis specimens showed high identity in this genomic region. All four isolates sequenced in this study were identical, although they came from different dogs and they were also very similar (99.6 and 97.3%) to the two *D. canis* isolates registered in GenBank, which were from a Chinese dog.²⁹ These results suggest homogeneity of world *D. canis* populations. Although it is very difficult to establish the limits of the intraspecific divergence, in general it is considered that the interspecific genetic distance must be about 10 times larger than the intraspecific one. 38,39 According to this criterion, it seems evident that D. injai is a different species from D. canis. because the mean interspecific distance (23.3%) was approximately 14.5 times greater than the intraspecific distance found in D. canis (0.4-2.7%). In the phylogenetic study. D. iniai appeared even closer to D. folliculorum than to D. canis, a fact that merits further investigation. Other criteria also support the consideration of D. injai as a separate species. As described by Desch and Hillier, 18 both species are morphologically very distinct and also have different habitats, with D. injai being located mainly in the sebaceous glands and sebaceous ducts and D. canis being an inhabitant of the lumen of the hair follicles. From the clinical point of view, D. injai seems to be associated with a specific clinical presentation, seborrhoeic dermatitis in middle-aged dogs, with terriers being over-represented in the series of cases published to date. 16,19,20

The molecular studies indicated that the short-bodied *Demodex* mite observed in some dogs and unofficially named *D. cornei* is a morphological variant of *D. canis*. The sequence of the short-bodied *Demodex* mite showed a divergence with *D. canis* (from 0.4 to 2.2%) similar to intraspecies variation in *D.canis*. The sequence of the short-bodied *Demodex* mite isolates was 99.5% identical to one of the *D. canis* mites (*D. canis*-2). This finding lends partial

support to the hypothesis that there is a variation in the size of *D. canis* depending on several factors, such as body site or habitat in the skin (the most superficial being shorter). The fact that *D. canis* and the short-bodied mite are commonly detected together in the same animal adds support to this hypothesis. Nevertheless, it is a bit surprising that all three isolates with the short-bodied mite morphology shared exactly the same sequence. This may simply be a coincidence or it may suggest the existence of a subpopulation inside *D. canis* with specific morphological features and a specific haplotype. The number of individuals investigated was too small to reach a conclusion on this question.

New species or variants of *Demodex* mites are described in different mammals, including dogs. 40,41 It is reasonable to think that hundreds of *Demodex* species colonize the skin of mammals. *Demodex* mites so far have been shown to have high host specificity and several species can share the same host, although they probably inhabit different cutaneous niches. The present study recommends the combined use of morphological and molecular methods (16S mitochondrial rDNA) for speciation. In the future, extensive sequencing of *Demodex* mites from different mammals will allow further detailing of the phylogenetic tree of the genus *Demodex*, which is still at an embryonic stage. Furthermore, the present study opens the possibility of the development of PCR techniques aimed at detecting species of *Demodex* in biological samples in epidemiological and clinical investigations.

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7. STUDY 3

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Authors:

Ivan Ravera^{*}, Laura Altet[†], Olga Francino[†], Armand Sánchez[†], Wendy Roldán^{*}, Sergio Villanueva^{*}, Mar Bardagí^{*} and Lluís Ferrer^{*}

- * Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.
- † Servei Veterinari de Genètica Molecular, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.

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Small *Demodex* populations colonize most parts of the skin of healthy dogs.

Abstract

It is unproven that all dogs harbour *Demodex* mites in their skin. In fact, several microscopic studies have failed to demonstrate mites in healthy dogs. We hypothesized that *Demodex canis* is a normal inhabitant of the skin of most. if not all, dogs. This hypothesis was tested using a sensitive real-time PCR to detect Demodex DNA in the skin of dogs. For this purpose, one hundred dogs living in a humane society shelter, 20 privately owned and healthy dogs and eight dogs receiving immunosuppressive or antineoplastic therapy. Hair samples (250-300 hairs with their hair bulbs) were taken from five or 20 skin locations. A real-time PCR that amplifies a 166-bp sequence of the D. canis chitin synthase gene was used. The percentage of positive dogs increased with the number of sampling points. When a large canine population was sampled at five cutaneous locations, 18% of dogs were positive for *Demodex DNA*. When 20 skin locations were sampled, all dogs tested positive for mite DNA. Our study indicates that *Demodex* colonization of the skin is present in all dogs, independent of age, sex, breed or coat. Nevertheless, the population of mites in a healthy dog appears to be small. Demodex DNA was amplified from all 20 cutaneous points investigated, without statistically significant differences. In conclusion, using a real-time PCR technique, Demodex mites, albeit in very low numbers, were found to be normal inhabitants of haired areas of the skin of healthy dogs.

Introduction

Understanding canine demodicosis remains one of the most difficult challenges in veterinary dermatology. Despite the prevalence and severity of the disease, many aspects of the pathogenesis of this entity remain obscure or poorly documented. Reference textbooks repeatedly make the following two statements:^{1,2} (i) *Demodex* mites are part of the normal fauna of the dog, and mites are present in the hair follicles of healthy dogs; and (ii) a genetically preprogrammed immunological defect is responsible for the exaggerated replication of mites in demodicosis.¹

The 'fact' that all dogs harbour *Demodex* mites in the skin has not been proved using reproducible scientific methods. Most textbooks refer to the classic research done by Gaafar et al., in which *Demodex* mites were found in the skin of 5.4% of healthy dogs. The authors concluded that *Demodex* mites could be found in the skin of healthy dogs and that 'follicular mange' is a complex condition, but not that all dogs harbour mites in their skin. More recently, Fondati et al., using trichoscopy, could not detect *Demodex canis* mites in any of 78 dogs examined, and found a single *Demodex* injai mite in one dog. Furthermore, aspects such as the preferred anatomical location of the mites on the canine skin, the mite density, and the influence of age and breed on the *Demodex* mite population remain unknown. Recently, we have developed a

highly sensitive real-time PCR to detect *D. canis* DNA.⁶ In that study, we were able to amplify the DNA of *D. canis* in nine of 51 dogs (17.6%), after sampling only two points of the skin (lateral face and interdigital skin).⁶ Interestingly, this is a much higher percentage than previously reported.^{7,8}

The prevalence of *Demodex* mites in the skin of healthy humans is close to 100%, with a mean mite density of 0.7 mites/cm2 (facial skin). ^{9–14} *Demodex* mites are assumed to be normal inhabitants of the skin of most mammals. ^{9,14,15} Therefore, considering data from other species and the biology of *Demodex* mites, the goal of the present study was to determine whether *D. canis* mites are normal inhabitants of the skin of most, if not all, dogs or if only a subset of the canine population harbours the mites, and these dogs serve as a reservoir of the parasite.

Materials and methods

Dogs

One hundred dogs living in a humane society shelter were used in this study. All were adult dogs, ranging in age from 9 months to 13 years, and included dogs of both sexes and of different breeds, with a large majority of dogs being of mixed breed. The shelter housed between 250 and 300 dogs, and only dogs with normal physical and dermatological examinations were used. As canine leishmaniosis is endemic in the region, serological testing was performed on all 100 dogs, and 17 tested positive. Dogs were sampled on three occasions, for experiments 1, 2 and 3. The Board of the Centre gave written permission for the study to be carried out, provided that that all sampling procedures were done under the supervision of the shelter veterinarians.

Twenty privately owned healthy adult dogs presented to the Veterinary Teaching Hospital for preventive medicine examinations and veterinary counselling were included in the study. In addition, eight dogs being treated at the Veterinary Medical Teaching Hospital for different diseases that required immunosuppressive or antineoplastic therapy for more than 2 months were also sampled. None of these eight dogs had skin lesions suggestive of demodicosis. All owners were informed of the nature of the study and gave their written consent.

Hair sampling and DNA extraction

Hair samples (n = 250–300) were obtained by gentle plucking of hair in the direction of the growth so as to include the hair bulb (root) in the sample. Each sample included 250–300 hairs. The number of sampling locations in each experiment is shown in Table 1. Hair samples were maintained in phosphate-buffered saline and stored at -20°C until DNA extraction. For the DNA extraction, samples were centrifuged in a microcentrifuge at maximal speed for 30 min; once the supernatant was removed, 200 μ L of digestion buffer (50 mmol/L Tris–HCl, pH 8.5; and 1 mmol/L EDTA) and 4 μ L of proteinase K solution (10 mg/ mL) were added, and samples were incubated at 56°C overnight. After inactivation of the proteinase K for 10 min at 95°C, the samples were centrifuged for 10 min at maximal speed. Supernatant was transferred to a new tube and diluted 1:10 for PCR amplification.

Experiments

Five different sampling experiments were conducted (Table 1). Briefly, in experiment 1, 100 healthy dogs living in an animal shelter had hair samples collected from the following five cutaneous locations: head, dorsal area, foreleg, abdomen and hindleg. In experiment 2, 16 dogs with positive samples from experiment 1 and 30 dogs with negative samples were resampled 6 months after the initial sampling. In experiment 3, five healthy dogs living in the shelter were sampled from the following 20 cutaneous locations: lip and periocular skin (four points), perinasal skin, temporal area, chin, ventral and dorsal neck, dorsum (two points), sternum, abdomen (two points), thigh (two points) and interdigital area (four points, one on each foot). As shelter dogs may not be representative of a normal canine population, five healthy, privately owned dogs were sampled in a similar manner (experiment 4). Finally, in experiment 5, eight dogs receiving immunosuppressive or antineoplastic therapy were sampled from five cutaneous locations.

PCR technique to detect Demodex DNA in canine hairs

The technique has been described in detail elsewhere. 6 Real-time PCR was carried out in a final volume of 20 µL using FastStart Universal SYBR Green Master (Roche Diagnostics GmbH, Mannheim, Germany), 0.3 µmol/L of each primer and 4 µL of diluted DNA. Primer pairs used were as follows: D. canis forward, 5'-GATGAAGCGGCGAGTAATGTTC-3'; and D. canis reverse, 5'-GACTCCATCTTTTACGATGTCTGATTT-3'. amplified Thev fragment of the chitin synthase gene. The eukaryotic 18S RNA Pre-developed TagMan Assay Reagent (Applied Biosystems, Foster City, CA, USA) was used as an internal reference for dog genomic DNA amplification to ensure suitability of each sample for PCR amplification and to be certain that negative results corresponded to true negative samples rather than to a problem with DNA loading, sample degradation or PCR inhibition. Water was used as a negative control for the PCR. Positive PCR controls were obtained from clinical samples that had previously been amplified and sequenced to confirm Demodex. The thermal cycling profile was 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Specificity assessment of the PCR was performed by adding a dissociation curve analysis at the end of the run. The product of the real-time PCR was sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Life Technologies Corp., Carlsbad, CA, USA) with the same primers, and the sequences obtained were compared with the GenBank database (http://blast.ncbi.nlm.nih. gov/Blast.cgi).

Statistical analyses

The chi-square test and the Saphiro–Francia normality test were used for the statistical analysis of the results ('R' program; R Foundation for Statistical Computing, Vienna, Austria).

Table 1. Dogs included in each experiment and results of the real-time PCR for *Demodex canis* DNA.

Experiment no.	Number of dogs and origin	Sampling locations	Number of dogs testing positive in Demodex PCR (%)
Experiment 1	100 healthy dogs from a shelter	5	18 (18%)
Experiment 2	16 dogs positive in experiment 1	5	3 (18.5%)
	30 dogs negative in experiment 1		5 (20%)
Experiment 3	5 healthy dogs from a shelter	20	5 (100%)
Experiment 4	20 privately owned dogs from VTH-UAB	20	18 (90%)
Experiment 5	8 dogs receiving immunosuppressive or antineoplastic therapy from the VTH-UAB	5	4 (50%)

Abbreviation: VTH-UAB, Veterinary teaching hospital – Universitat Autónoma de Barcelona.

Results

The results of the five experiments are shown in Table 1. Briefly, the percentage of positive dogs increased with the number of sampling sites. When a large canine population (experiment 1) was sampled at five cutaneous locations, 18% of the dogs were positive for *Demodex* mites using PCR. No association between sex, age, and type of coat or *Leishmania* seropositivity was found. The percentage of *Leishmania* seropositive dogs was similar in the two groups (22.2% in the positive group and 15.8% in the negative group; P > 0.1, chi-square test).

This relatively low percentage of positive dogs could indicate that only a subset of the canine population harbours Demodex mites, acting as a reservoir of the parasite, as mentioned in the introduction. However, the low percentage could also be due to low diagnostic sensitivity of the technique used, which resulted in Demodex not being detected in some or most dogs. To resolve this dilemma, we repeated the sampling 6 months later (experiment 2), including dogs that tested positive and negative in the first trial, to verify that positive dogs (n = 16) remained positive and negative dogs (n = 30) remained negative. In this second experiment, only three of the 16 previously positive dogs tested positive (18.75%), but five of the 30 previously negative dogs (20%) tested positive at one or more sampled site. A possible explanation for this was low test sensitivity, resulting in some negative results being false negatives (i.e. some dogs had Demodex mites in their skin but the mites were probably not included in the samples). Experiment 3 was conducted to increase the number of sampling sites in order to determine whether the percentage of positive dogs also increased. When dogs were sampled at 20 skin locations, all five dogs tested positive in at least one location; increasing the sample size increased the sensitivity, as the percentage of positive dogs was 100% (five of five).

The results of experiment 4 (privately owned dogs) confirmed that the majority of the healthy dogs (18 of 20) harboured *Demodex* mites in the skin. In this last group of dogs, *Demodex* DNA could be amplified from all of the 20 skin locations in at least one of the dogs (range one to six positive dogs at each

location). No individual sample site had significantly more positive outcomes than others (P > 0.1, Shapiro–Francia normality test).

Finally, we investigated a group of eight dogs receiving immunosuppressive or antineoplastic therapy (experiment 5). In this group, the percentage of positive dogs was clearly higher than in the general canine population. Four of the eight dogs (50%) were positive after sampling only five sites (general canine population 18%), and in three of the dogs three or more locations were positive (Table 2).

Table 2. Data of the dogs included in experiment 5 and results of the real-time PCR for *Demodex canis* DNA.

Signalment and diagnosis	Treatment	Result of the <i>Demodex</i> PCR
Boxer dog, male, 2 years old; corticosteroid- responsive meningitis	Prednisone, 2 mg/kg/day; 2 months	Positive for 3 of 5 skin points
Mixed breed, female, 7 years old; immune-mediated haemolytic anaemia	Prednisone, 1-2 mg/kg/day; 2 months	Negative (only four points sampled)
Golden retriever dog, male, 10 years old; multiple myeloma	Melphalan, prednisone (1 mg/kg/day), famotidine; 3 months	Negative
German shepherd dog, male, 4 years old; immune-mediated haemolytic anaemia	Prednisone, 1-2 mg/kg/day; and azathioprine, 1 mg/kg; 2 months	Negative
Labrador retriever dog, male, 5 years old; immune-mediated polyarthritis	Prednisone, 1–2 mg/kg/day and ciclosporin, 5 mg/kg; >3 months	Positive for 1 of 5 skin points
Bull mastiff dog, female, 6 years old; lymphoma (stage IV)	Madison–Wisconsin protocol* (prednisone from 2 to 0.5 mg/kg/day); 6 weeks	Positive for 5 of 5 skin points
Mixed breed, male, 9 years old; splenic haemangiosarcoma	Doxorubicin, chlorambucil; 2 months	Positive for 3 of 5 skin points
German shepherd dog, female, 4 years old; lymphoma (stage IVb)	Madison–Wisconsin protocol* (prednisone from 2 to 0.5 mg/kg/day); 6 weeks	Negative

^{*}Madison-Wisconsin protocol includes L-asparaginase, vincristine, cyclophosphamide, doxorubicin and prednisone.

Discussion

From this study, it was difficult to demonstrate that *Demodex* mites lived in the skin of all dogs. However, we were able to amplify *D. canis* DNA from the skin of most healthy dogs. Therefore, *Demodex* can be considered a normal inhabitant of the canine skin. This seems to be the case for most mammals investigated so far.¹⁴ In humans, for instance, the prevalence of *Demodex* mites on the facial skin, especially on the chin, is reported to be close to 100%.^{9–11} Our study indicates that *Demodex* colonization of the skin is present in dogs, independent of age, sex, breed or coat.

Nevertheless, the population of mites in a healthy dog must be very small, for several reasons. First, in clinical practice positive skin scrapings and skin biopsy samples that identify *Demodex* mites are rare in healthy dogs. Second, exhaustive rigorous microscopic examination of 78 dogs failed to detect a single *D. canis* mite.⁵ Third, even using a highly sensitive real-time PCR technique, we had to increase the number of sampling sites to be able to detect the parasite in a majority of dogs. In humans, however, the estimated mean mite density is as high as 0.7 to over 5 mites/cm² in some disease entities, such as rosacea and perioral dermatitis.^{10,13} The current technique, unfortunately, does not allow quantification of the number of mites in the sample, although development of a modified quantitative technique is underway.

Interestingly, *Demodex* DNA was amplified from all the cutaneous locations investigated, without statistically significant differences. The chin and perilabial skin locations were positive more frequently than the interdigital and perianal skin, but the differences were not significant. This is in contrast with the situation in humans, where *Demodex* is located almost exclusively in some parts of the facial skin. These results also differ somewhat from those of the pioneering study of Greve and Gaafar,⁴ who detected *D. canis* in all sampled areas of the skin, but more abundantly on the head and legs. According to our data, dogs harbour a very small population of *Demodex* mites in all sites of haired skin that were sampled, and these mites probably maintain nomadic behaviour on the skin surface. It is well known that *Demodex* mites move at a speed of 16 mm/h and move from one follicle to the next,⁹ especially during the night due to their negative phototaxia.

The *Demodex* colonization of the skin of mammals seems to be an extraordinary example of adaptation of one organism to another. Some authors consider that rather than parasitism, this should be viewed as commensalism or a mutualistic relationship. Our present knowledge suggests that *Demodex* mites normally have a symbiotic relationship with mammals. In normal circumstances, they appear to live as commensals, feeding on their host's sebum. It is possible in this role that they may even confer a mutualistic host benefit by ingesting bacteria or other organisms in the follicular canal. He host's innate immune system appears to tolerate the presence of these mites, but it may have a 'culling' or inhibitory effect on mite proliferation, keeping numbers in the hair follicle under control without inducing an inflammatory response. If mite numbers increase to a critical level (possibly causing physical distension of follicles with keratinocyte disruption), they could develop

a pathogenic role, causing insult to the host. 16,18-20 Furthermore, in most mammals *Demodex* behaves as an opportunistic pathogen with the potential to change its status from commensal to parasite (the mites benefit but harm the host) if the host's cutaneous environment facilitates their proliferation. In this context, the dog seems to be unique, with a small number of *Demodex* mites on healthy skin, but a high prevalence of cases of generalized and severe demodicosis in young dogs, without a primary or predisposing cause. The most plausible explanation is that canine generalized juvenile demodicosis is one of the negative traits associated with the creation of dog breeds. This would explain the strong breed predisposition for demodicosis, with the odds ratios for some breeds being as high as 35.5 (American Staffordshire terrier), 17.1 (Staffordshire bull terrier), 7.2 (Chinese shar-pei) and 5 (French bulldog). In addition, the genetic association between demodicosis and some alleles of the dog leukocyte antigen system has been reported. The genetic aspects of canine demodicosis require urgent investigation.

Demodicosis associated with in humans is treatment with immunosuppressive drugs^{13,24–26} and with some antineoplastic therapies, such as epidermal growth factor receptor inhibitor.²⁷ Furthermore, one of the main causes of generalized demodicosis in adult dogs is prolonged corticosteroid therapy. We therefore investigated the presence of *Demodex* DNA in the skin of a group of dogs receiving corticosteroid therapy or antineoplastic therapy. This preliminary study seems to demonstrate an increase in the presence of Demodex mites in these patients. Although these results must be confirmed in a larger study, they seem to suggest that some immunosuppressive therapies induce a progressive increase of the cutaneous *Demodex* populations resulting in clinical demodicosis.

In short, the present study formally demonstrates, by means of a real-time PCR technique, that *Demodex* mites, albeit in very low numbers, are normal inhabitants of hairy skin of healthy dogs.

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8. STUDY 4

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Authors:

Natalia Sastre^{*}, Ivan Ravera[†], Diana Ferreira[†], Laura Altet^{*}, Armand Sánchez^{*}, Mar Bardagí[†], Olga Francino^{*}, and Lluís Ferrer[‡].

- * Servei Veterinari de Genètica Molecular, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.
- † Department of Animal Medicine and Surgery, Veterinary School, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.
- ‡ Department of Clinical Sciences, Cummings School of Veterinary Medicine, Tufts University, 01536 North Grafton, MA, USA.

Development of a PCR technique specific for *Demodex injai* in biological specimens.

Abstract

The identification of *Demodex injai* as a second *Demodex* species of dog opened new questions and challenges in the understanding on the Demodexhost relationships. In this paper, we describe the development of a conventional PCR technique based on published genome sequences of D. injai from GenBank that specifically detects DNA from D. injai. This technique amplifies a 238-bp fragment corresponding to a region of the mitochondrial 16S rDNA of D. injai. The PCR was positive in DNA samples obtained from mites identified morphologically as D. injai, which served as positive controls, as well as in samples from three cases of demodicosis associated with proliferation of mites identified as D. injai. Furthermore, the PCR was positive in 2 out of 19 healthy dogs. Samples of *Demodex canis* and *Demodex folliculorum* were consistently negative. Skin samples from seven dogs with generalized demodicosis caused by *D. canis* were all negative in the *D. injai*-specific PCR, demonstrating that in generalized canine demodicosis, mite proliferation is species-specific. This technique can be a useful tool in the diagnosis and in epidemiologic and pathogenic studies.

Introduction

Demodex mites are considered normal inhabitants of the skin of mammals. Currently, 88 species of *Demodex* have been described from 83 mammal species, with over half the mite species from just two host orders, the rodents (28 mite species from 29 host species), and the chiropterans (17 mite species from 13 host species). *Pemodex canis* was considered for a long time to be the sole *Demodex* species on the dog. Canine generalized demodicosis is considered to be a consequence of a severe overgrowth of *D. canis* mites due to a still poorly characterized hereditary immunodeficiency or acquired immunosuppression. However, in the late 1990s, several authors described a different demodecid mite in dogs, characterized by a long opisthosoma. This species was morphologically characterized and named *Demodex injai* by Desch and Hillier. Later, several authors described dermatologic conditions, mainly seborrheic dermatitis in terrier dogs, associated with *D. injai* overgrowth.

Sequencing the mitochondrial 16S rDNA demonstrated that *D. injai* is genetically different from *D. canis* and is probably closer to the human mite *Demodex folliculorum*.¹³ However, most aspects of the biology of *D. injai* remain unknown. *D. injai* is considered a normal inhabitant of canine skin and has occasionally been detected in healthy dogs,¹⁴ and some authors suggest that these mites live mostly in the sebaceous glands and sebaceous ducts, rather than in hair follicles,¹⁵ as *D. canis* does.

Furthermore, the presence of two or more *Demodex* species in one host poses a new and interesting question, *i.e.*, whether both species proliferate in demodicosis. Seen from another perspective, the question is whether the defect leading to the mite overgrowth is species-specific or not.

To advance understanding on the biology of *D. injai* and of canine demodicosis, we designed primers based on the published sequences of mitochondrial 16S rDNA that specifically amplify *D. injai* DNA. Subsequently, we performed a conventional PCR technique on different skin and hair samples of healthy dogs and of dogs with demodicosis.

Materials and methods

Samples

Forty hair samples from nineteen dogs living in a humane society shelter that were included in a previous study¹⁶ were used to investigate the presence of D. injai in normal dogs. The Board of the Centre gave written permission for the study to be carried out, provided that all sampling procedures were done under the supervision of the shelter veterinarians. Hair samples were obtained by gentle plucking of hair in the direction of the growth so as to include the hair bulb (root) in the sample. Each sample included 250-300 hairs. The sampling sites were the head, dorsum, lumbar skin, feet, and abdomen. These samples had yielded a positive result in real-time PCR for *Demodex* chitin synthase, and therefore, they were considered to contain *Demodex spp.* DNA. Skin scrapings of seven dogs diagnosed with juvenile generalized demodicosis due to D. canis and skin scrapings of three dogs with a diagnosis of seborrhoea associated with D. injai overgrowth were also included in the study. In none of the samples both mite species (D. canis and D. injai) were simultaneously detected. Both D. canis and D. injai mites were identified microscopically, after measuring the gnathosoma, podosoma, and opisthosoma, according to the original descriptions. ^{5,10} Hair samples and skin scrapings were conserved in physiological saline solution and stored at -20 °C until DNA extraction. In the case of privately owned dogs, all the owners were informed on the nature of the study and gave their written consent.

DNA extraction

For the DNA extraction, frozen hair samples or skin scrapings in physiological saline solution were thawed and centrifuged in a microcentrifuge at $16,000\times g$ for 30 min. Once the supernatant was removed, 200 µL of digestion buffer (50 mmol/L Tris–HCl, pH 8.5; and 1 mmol/L EDTA) and 4 µL of proteinase K solution (10 mg/mL, Roche Applied Science) were added, and samples were incubated at 56 °C overnight. After inactivation of the proteinase K for 10 min at 95 °C, the samples were centrifuged for 10 min at $16,000\times g$. Supernatant containing the *Demodex* DNA was transferred to a new tube and diluted 1:10 for real-time PCR amplification.

DNA from individual *Demodex* mites that were aspirated using a micropipette and identified morphologically as *D. canis*, *D. injai*, and *D. folliculorum* served as positive controls. These mites had previously been

identified as *D. canis*, *D. injai*, and *D. folliculorum*, respectively, after amplifying and sequencing a fragment of the mit16S RNA as described in Sastre et al.¹³

Real-time PCR for Demodex chitin synthase

The technique has been described in detail elsewhere 17 and amplifies a 166-bp fragment of the chitin synthase gene of *D. folliculorum*, *D. canis*, and *D.* injai. Real-time PCR was carried out in a final volume of 20 µL using FastStart Universal SYBR Green Master (Roche Diagnostics GmbH, Mannheim, Germany), 0.3 µmol/L of each primer, and 4 µL of diluted DNA. Primer pairs used were as follows: Demodex forward, 5'-GATGAAGCGGCGAGTAATGTTC-3', and *Demodex* reverse, 5'-GACTCCATCTTTACGATGTCTGATTT-3'. They amplified a 166-bp fragment of the chitin synthase gene. The eukaryotic 18S RNA Pre-developed TagMan Assay Reagent (Applied Biosystems, Foster City, CA, USA) was used as an internal reference for dog genomic DNA amplification to ensure suitability of each sample for PCR amplification and to be certain that negative results corresponded to true negative samples rather than to a problem with DNA loading, sample degradation, or PCR inhibition. The thermal cycling profile was 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Specificity assessment of the PCR was performed by adding a dissociation curve analysis at the end of the run. Realtime PCR products were sequenced with the BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit, version 3.1 (Life Technologies Corp., Carlsbad. CA, USA), with the same primers, following the manufacturer's protocol. Sequences were purified using the Montage SEQ96 Sequencing Reaction Cleanup Kit (Millipore, MA, USA) and separated on an ABI PRISM 3730 automated sequencer (Applied Biosystems) according to the protocol provided by the manufacturer. All sequences were examined with SEQSCAPE 2.1.1 (Life **Technologies** Corp.) and compared with GenBank database the (http://www.ncbi.nlm.nih.gov/BLAST).

PCR for D. injai and sequencing

PCR was carried out in a final volume of 20 µL containing 1 µL of DNA solution, PCR buffer (1x), 1.9 mM MgCl₂, 0.1 mM of each dNTP, 0.5 µM of each primer, and 1 U AmpliTag Gold DNA Polymerase (all from Applied Biosystems. Foster City, CA, USA). Primer pairs used were as follows: D. injai forward, 5'-AATGAAGGGCTTTTGTTGAACCTA-3' and D. injai reverse. AAAAATAATCCTGTTACCCCCAAA-3'. They amplified a 238-bp fragment of the mitochondrial 16S rDNA of *D. injai*. The thermal cycling profile was 95°C for 10 min, followed by 40 cycles at 94°C for 30 s, 58.5°C for 30 s, and 72°C for 30 s. Water negative controls were run with every PCR. Real-time PCR products were sequenced with the BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit, version 3.1 (Life Technologies Corp., Carlsbad, CA, USA), with the same primers, following the manufacturer's protocol. Sequences were purified using the Montage SEQ96 Sequencing Reaction Cleanup Kit (Millipore, MA, USA) and separated on an ABI PRISM 3730 automated sequencer (Applied Biosystems) according to the protocol provided by the manufacturer. All sequences were examined with SEQSCAPE 2.1.1 (Life Technologies Corp.) compared with the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST).

Results

The real-time PCR for *Demodex* chitin synthase amplified the expected 166-bp segment from the DNA obtained from *D. canis*, *D. injai*, or *D. folliculorum*. In contrast, the classical PCR for *D. injai* was positive only in the control samples that contained DNA extracted from mites identified as *D. injai* morphologically, and it was negative in the samples of DNA from *D. canis* and *D. folliculorum*, confirming the specificity of the assay for *D. injai*. As expected, the technique amplified a 238-bp segment, and its sequence matched in the GeneBank (accession numbers JX193757, JX390980) with the mitochondrial 16S rDNA of *D. injai* (Figure 1).

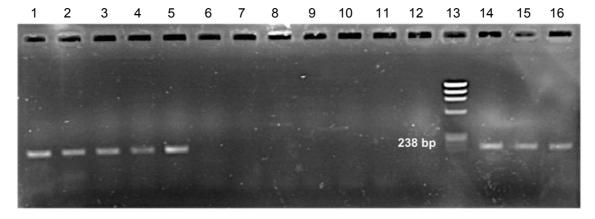
Four of the 40 hair samples from healthy dogs were also positive in the PCR for *D. injai*. Three samples belonged to one dog and one to a second dog; therefore only 2 out of the 19 dogs were positive. All these four samples had also been positive for the real-time PCR for *Demodex* chitin synthase.

All three samples obtained from the skin scrapings from dogs with a clinical and parasitologic diagnosis of demodicosis caused by *D. injai* were positive for *D. injai*-specific PCR. In contrast, all seven samples obtained from dogs with a clinical and parasitological diagnosis of demodicosis caused by *D. canis* were negative for *D. injai*-specific PCR amplification (Figure 2).

Figure 1. Alignment of the 16S rDNA fragments of *D. injai*, *D. canis*, and *D. folliculorum* (JX390978 and JF783994 were retrieved in the GenBank; the *D. injai* fragment (suffix "UAB") was sequenced in the present study.

D. injai_UAB	ACTTGTATGA GGGGAAA-A						
D. canis_JX390978	TGTT	G.G.TTT	AAG	GTTT.GC	T	AC	[70]
D. folliculorum_JF783994	TGTA	G.C	ATT	GCA	T	A	[70]
D. injai_UAB	AAATTTAGAA GTTTTTTCA						
D. canis_JX390978	GT T.G		GT		AAAA.A.GGG	TTT	[140]
D. folliculorum_JF783994	G		T	• • • • • • • • • • • • • • • • • • • •	AGG	.TT	[140]
D. injai_UAB	TATTTGGGGG AAAGGGTAA	T TAGTGTTAAT	TGTTTTTTTA	TATTGAACTT	TGGTAGGTTA	AGTGGATAGG	[210]
D. canis_JX390978	.GA.T	TAT.	.TC	GTG	T	.AA	[210]
D. folliculorum_JF783994	T						
D. injai_UAB	TACTTTGGGG GTAACAGG						
D. canis_JX390978	C T						
D. folliculorum_JF783994		c	. [241]				

Figure 2. Results of the PCR to amplify 16S rDNA from Demodex injai.



Lanes 1 and 2: DNA extracted from mites identified as *D. injai* (positive controls). Lanes 3, 4, and 5: skin scrapings from dogs diagnosed with demodicosis caused by *D. injai*. Lanes 6–11: skin scrapings from dogs with JGD. Lane 12: negative control (water). Lane 13: marker. Lanes 14–16: samples from a healthy dog previously positive to PCR chitin synthase.

Discussion

This paper describes a new PCR technique to detect DNA specific to *D. injai* in biological samples. This PCR amplified a 238-bp DNA fragment in all *D. injai* samples and was consistently negative in all *D. canis* samples. The technique is based on published sequences of the mitochondrial 16S ribosomal DNA of *D. injai* and can be useful in future epidemiologic and pathogenic studies, and also as a diagnostic tool.

Very little is known about the biology of *D. injai*. This pilot study confirms a previous observation of *D. injai* in a healthy dog.¹⁴ Two dogs out of 19 were positive for *D. injai*, suggesting that *D. injai* could be part of the normal fauna of some dogs, although the populations of mites are probably also small, as is the case with *D. canis*.¹⁶ Nevertheless, a larger epidemiologic study is necessary to learn whether the parasite is present in all dogs or only in a subpopulation of dogs and also to learn what the anatomic distribution on the canine skin is.

Interestingly, all samples from dogs with generalized canine demodicosis associated with *D. canis* overgrowth were negative for *D. injai*. Canine generalized demodicosis is considered to be the consequence of a genetic defect in the control of *Demodex* populations. If *D. injai* is a normal inhabitant of canine skin, at least in some dogs, it would be reasonable to expect to find *D. injai* overgrowth together with *D. canis* mites. Our results, in contrast, suggest that in cases of canine generalized demodicosis, only *D. canis* mites proliferate. The genetic defect leading to this proliferation seems to affect only the control of *D. canis* populations. At present, it is not possible to find a mechanistic explanation for this finding. It would be of interest to investigate whether this is also the case in other species with two *Demodex* species (e.g., *D. folliculorum* and *D. brevis* in humans with rosacea). These results, then, underline the need for deeper understanding on the mechanisms of the host–*Demodex* relationship.

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9. STUDY 5

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Authors:

Ivan Ravera^{*}, Diana Ferreira^{*}, Laia Solano Gallego^{*}, Mar Bardagí^{*}, Lluís Ferrer[†]

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^{*} Department of Animal Medicine and Surgery, Veterinary School, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.

[†] Department of Clinical Sciences, Cummings School of Veterinary Medicine, Tufts University, 01536 North Grafton, MA, USA.

Serum detection of IgG antibodies against *Demodex canis* by western blot in healthy dogs and dogs with juvenile generalized demodicosis.

Abstract

The aim of this study was to investigate the presence of canine immunoglobulins (Ig) G against *Demodex* proteins in the sera of healthy dogs and of dogs with juvenile generalized demodicosis (CanJGD) with or without secondary pyoderma. *Demodex* mites were collected from dogs with CanJGD. Protein concentration was measured and a western blot technique was performed. Pooled sera from healthy dogs reacted mainly with antigen bands ranging from 55 to 72 kDa. Pooled sera from dogs with CanJGD without secondary pyoderma reacted either with 10 kDa antigen band or 55 to 72 kDa bands. Pooled sera from dogs with CanJGD with secondary pyoderma reacted only with a 10 kDa antigen band. The results of this study suggest that both healthy dogs and dogs with CanJGD develop a humoral response against different proteins of *Demodex canis*.

Introduction

Canine juvenile generalized demodicosis (CanJGD) is an inflammatory skin disease associated with overpopulation of the normal hair follicle mite *Demodex canis*, and in some cases, *Demodex* injai.¹⁻⁴ CanJGD is considered a hereditary skin disease due to its high prevalence in purebred young dogs, although its mode of inheritance and genetic defect(s) remain unknown.^{2,5-7}

Although the immune system is considered to play a key role in the pathogenesis of canine demodicosis, 6,8-10 the mechanisms that control mite populations in healthy and diseased dogs remain far from being understood. Most authors agree that the immune response against *Demodex* mites is complex and involves both branches of host immune response: innate and adaptive response. Innate immune response, considered the first line of defence, has been poorly investigated in human and canine demodicosis, but some evidence suggested that chitin-bearing organisms could modulate the innate immune response by increasing Toll-like receptor expression. 11 During the past four decades, authors have focused in the cellular immunity, and studies have suggested that the main mechanism of control of mite population would be cell-mediated. 12-16 On the contrary, very few studies have been conducted to investigate the eventual existence of a humoral immune response against Demodex mites. The results of these studies were scarce and inconclusive, and it was assumed that humoral response did not play a role in the control of mite populations.² The lack of a standardized or commercial Demodex antigen/extract may explain the paucity of experimental studies on the humoral response against Demodex mites. On the other hand, in other canine ectoparasitic skin diseases such as scabies, this problem has been widely overcome. 17-19 In order to enlighten the field of humoral response in canine demodicosis, the purposes of the present study were (1) to obtain a whole body *D. canis* crude protein extract, and (2) investigate the presence of humoral response against *D. canis* proteins in dogs with CanJGD with or without secondary pyoderma and in healthy dogs sera.

Materials and methods

Dogs

Thirty-one client-owned dogs with no history of sarcoptic mange and cutaneous hypersensitivities were diagnosed with CanJGD. Diagnosis was made by the detection of mature and immature D. canis mites in scrapings and/or hair plucking samples from lesional skin. Blood samples were taken from theses affected dogs. Diagnosis of concurrent secondary pyoderma was made by cytologic examination of several papules/pustules. When skin cytology revealed the presence of neutrophils and intracellular cocci, a diagnosis of concurrent secondary pyoderma was made. The presence of *Malassezia spp*. overgrowth by skin cytology was not recorded. The mean age of dogs with CanJGD was 12 months (4 to 108 months-old) and the breeds included were: pug (n = 7), boxer (n = 6), French bulldog (n = 3), Yorkshire terrier (n = 3), dogue de Bordeaux (n = 2), crossbreed (n = 2), English bull- dog (n = 1), golden retriever (n = 1), American Staffordshire terrier (n = 1), doberman pinscher (n = 1), German shepherd (n = 1), Bernese mountain dog (n = 1), miniature pinscher (n = 1), and German shorthaired pointer (n = 1). Fourteen of the 31 patients with CanJGD (14/31) were considered as dogs without concurrent secondary pyoderma. Skin cytology revealed the presence of concurrent secondary pyoderma in 17/31 dogs.

Healthy dog blood sample were obtained from 19 dogs that were admitted to the Universitat Autònoma de Barcelona Veterinary Teaching Hospital (UAB-VTH) for regular health-check, pre-neutering check-up, vaccination, and regular de-worming, after obtaining written permission from the owners. None of the healthy dogs included in this study had a previous history of sarcoptic mange or cutaneous hypersensitivity. The mean age of healthy dogs was 43 months (8 to 108 months-old) and the breeds included were: crossbreed (4), Labrador retriever (n = 2), Golden retriever (n = 1), English setter (n = 1), German shepherd (n = 1), Catalan shepherd dog (n = 1), Brittany spaniel (n = 1), bullmastiff (n = 1), Belgian shepherd dog (n = 1), English bulldog (n = 1), dachshund (n = 1), Dalmatian (n = 1), border collie (n = 1), Shetland sheepdog (n = 1), and poodle (n = 1).

All blood samples were centrifuged at 1.500 rpm during 10 min. Sera were extracted and transferred into an eppendorf tube. Sera were divided into 3 groups: healthy group, CanJGD without secondary pyoderma group, and CanJGD with secondary pyoderma group. In addition, sera from the healthy group were randomly subdivided into 4 groups, CanJGD sera without concurrent secondary pyoderma were randomly subdivided into 3 groups, and CanJGD sera with concurrent secondary pyoderma were randomly subdivided into 4 groups. Each subgroup contained 4 to 5 polled sera. All sera and pooled sera were stored at -80 °C.

Preparation of a whole body D. canis crude protein extract

Skin scrapings were performed on 50 patients with a diagnosis of CanJGD visited at the UAB-VTH. After scraping, samples were transferred into a sterile glass slide containing a drop of glycerol. For mite collection, skin scrapings were transferred into a tube and mixed with phosphate buffered saline (Phosphate buffered saline tablet; Sigma-Aldrich Química; Madrid; Spain). Mixture was then placed on an 11 µm nylon net filter (Millipore Iberica SAU, Madrid, Spain) held in a stainless steel filter holder (Swinny®; Millipore Iberica SAU; Madrid; Spain). The sample was washed 3 times and filtered with phosphate buffered saline (PBS). The net filter was recovered from the filter and placed on top of a clean slide. The sample was observed with an optical microscope (10x). Each visualized *D. canis* mite was transferred from the net filter with a 30 gauges needle and placed into an eppendorf tube containing PBS. By this method, around 1200 mites were collected. Next, sample containing Demodex mites was disrupted with liquid nitrogen (Carburos Metálicos; Barcelona; Spain) in a mortar (Haldenwanger mortar; Fisher Scientific; Madrid; Spain) until obtaining a fine powder. Finally, the powder was diluted again in PBS.

Quantification of proteins

Protein extraction was supported by Protein Production Platform (CIBER-BBN-UAB, http://www.ciber-bbn.es/en/programas/89-plataforma-de-produccion-de-proteinas-ppp). A Bradford kit (QuickStartTM Bradford Protein Assay Kit; Bio-Rad Laboratories; Madrid; Spain) was used for the quantification of proteins. Measures were run in duplicates. The median concentration of protein extracted after the disruption was 6.8 ng/ μ L.

Detection of anti-D. canis IgG antibodies

Two hundred and fifty microliters of the extract sample were analysed by denaturing sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) at 10% acrylamide. The sample was re-suspended with denaturing buffer (Tris base 1.28 g, glycerol 8 ml, SDS 1.6 g, β-mercaptoethanol 4 ml, urea 9.6 g), 20 boiled for 10 min and loaded onto the gel. Electrophoresis was performed during 1,5 h at 100 V. Then, proteins were electroblotted (1 h at 100 V) onto nitrocellulose membranes. Blocking of non-specific binding sites was performed with powdered milk under gentle agitation overnight. Two hundred microliters of different groups of pooled sera were loaded in a system for cross-blot and incubated during 2 h at room temperature (RT) under gentle agitation. Two pooled sera titers were used: 1/200 and 1/1000. Membrane was washed twice with PBS + 0.5% Tween-20 (Tween®20, Sigma-Aldrich Química, Madrid, Spain). A rabbit anti-dog immunoglobulin G (IgG) horseradish peroxidase conjugated (Anti IgG [H&L] Dog PO, Rockland Immunochemicals Inc. Pennsylvania, United States) antibody was used as secondary antibody at a 1/10,000 dilution and incubated during 1 h at RT under gentle agitation. Membrane was washed 3 times with PBS + 0.5% Tween-20 during 15 min. Bands were detected by chemiluminescence using the SuperSignal West Pico Chemiluminiscent substrate (Thermo Scientific, Rockford, IL, United States) and images were taken with the Quantity One software (Bio-Rad Laboratories SA, Madrid, Spain).

Detection of anti-Dermatophagoides farinae IgG antibodies

In order to compare the previous experiment with a known commercial extract, a second western blot was performed using a lyophilized whole-body extract of *D. farinae* (Laboratorios Datier SA, Madrid, Spain) as the source of antigen. The concentration used was of 1 mg/mL. Procedure was identical to the previous experiment.

Results

Detection of anti-D. canis IgG antibodies

Bands were observed when dilution of pooled sera was used at 1/200 (Figure 1) while no bands were detected at 1/1000 sera dilution.

Sera from healthy dogs showed two common bands among 55 and 72 kDa (Fig. 1, lanes 1 to 4), a pattern also seen in one of the groups of dogs with CanJGD without secondary pyoderma (Fig. 1, lane 5). Sera from dogs with CanJGD without secondary pyoderma presented bands towards 10 kDa and 55 to 72 kDa. (Fig. 1, lanes 5 to 7). Sera from dogs with CanJGD with secondary pyoderma only presented bands towards 10 kDa. (Fig. 1, lanes 8 to 11).

A
B
C
D
E
F
G
H

Figure 1. Western blot with Demodex canis extract.

Lanes 1 to 11, correspond to pooled sera with a secondary antibody dilution of 1/200. Lanes 1 to 4: pooled sera from healthy dogs. Lanes 5 to 7: pooled sera from dogs with CanJGD without secondary pyoderma. Lanes 8 to 11: pooled sera from dogs with CanJGD with secondary pyoderma. Lanes 12 to 22, correspond to pooled sera incubated with a dilution of the secondary antibody of 1/1000. Marker: A, 150 kDa; B, 130 kDa; C, 95 kDa; D, 72 kDa; E, 55 kDa; F, 43 kDa; G, 34 kDa; H, 26 kDa; and I, 10 kDa.

11 12 13 14 15 16 17 18 19 20 21

Detection of anti-D. farinae IgG antibodies

Bands were observed when dilution of pooled sera was used at 1/200 (Figure 2) while no bands were detected at 1/1000 sera dilution.

Sera from healthy dogs presented bands above the 72 kDa (Fig. 2, lanes 1 to 4). In addition, the first group also presented a band around 55 kDa (Fig. 2, lane 1), the third group showed a band between 55 and 43 kDa (Fig. 2, lane 3), and the fourth group presented a band between 26 and 10 kDa (Fig. 2, lane 4). Sera from dogs with CanJGD without secondary pyoderma: only the first group of sera (Fig. 2, lane 5) presented a band of reaction above 72 kDa. Sera from dogs with CanJGD with secondary pyoderma: only the fourth group (Fig. 2, lane 11) presented one band around 10 kDa and one band between 26 and 10 kDa.

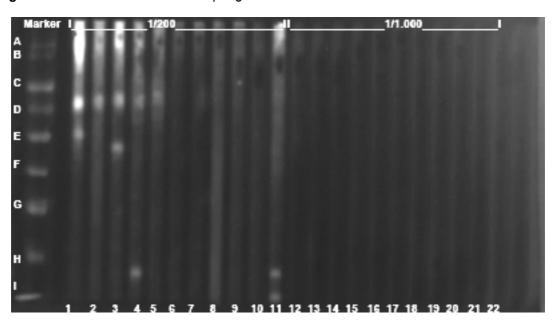


Figure 2. Western blot with Dermatophagoides farinae extract.

Lanes 1 to 11, correspond to pooled sera with a secondary antibody dilution of 1/200. Lanes 1 to 4: pooled sera from healthy dogs. Lanes 5 to 7: pooled sera from dogs with CanJGD without secondary pyoderma. Lanes 8 to 11: pooled sera from dogs with CanJGD with secondary pyoderma. Lanes 12 to 22, correspond to pooled sera incubated with a dilution of the secondary antibody of 1/1000. Marker: A, 150 kDa; B, 130 kDa; C, 95 kDa; D, 72 kDa; E, 55 kDa; F, 43 kDa; G, 34 kDa; H, 26 kDa; and I, 10 kDa.

Discussion

Although in previous studies, 14,21 Demodex proteins were used to investigate the role of antibodies in the pathogenesis of demodicosis, to the authors' knowledge, the results of the present study demonstrate for the first time the presence of IgG antibodies directed against *D. canis* antigens in the serum of healthy dogs and of dogs with CanJGD. Sera of healthy dogs and dogs with CanJGD without secondary pyoderma consistently reacted against

antigens of 55 kDa and 72 kDa, proving the existence of a humoral immune response against *Demodex* mites.

There are many evidences demonstrating that the immune system of the host is responsible for the control of cutaneous *Demodex* mite populations. There is, however, a paucity of information concerning the mechanism of the immune response against Demodex. Some authors have detected that the Tcell mediated immune responses are depressed in dogs with demodicosis, suggesting that T lymphocytes have a predominant role in the immune response against Demodex. There are much less data about the existence of a humoral immune response against the mites. Healey and Gaafar¹⁴ failed to demonstrate by radioimmunoelectrophoresis the presence of anti-mite immunoglobulins in the sera of demodectic dogs and specific-pathogen-free dogs. Grosshans et al.²¹ by different immunoserological methods demonstrated Demodex specific antibodies in the sera of people with rosacea, diseased goats, and of rabbits sensitized with small amounts of demodectic antigen. The results of the present study are relevant because demonstrate the existence of serum IgG antibodies against *D. canis*. Nevertheless, the significance of this humoral response remains be determined. It could be that the humoral immune response may be part of the immune mechanism that control Demodex populations in dogs. This could be supported by the unexpected findings of Liu et al. 22 using double knockout mouse for STAT6-/- and CD28-/-. This mouse strain has impaired humoral immune response because of the lack of STAT6, a signaling molecule for IL-4, and develops severe demodicosis at early age (3) months). Our results would also support that humoral response plays a role in the control of *Demodex* mites. However, it could also be that these antibodies represent only an epiphenomenon, non-relevant for the control of Demodex populations since in most clinical cases, dogs with CanJGD needs miticidal treatment for the resolution of the disease.²³

It is difficult to explain why dogs with demodicosis and secondary pyoderma present antibodies against a protein of different molecular weight (10 kDa). One possible explanation could be that the furunculosis induced by bacterial infection would lead to mite release into the dermis and the generation of a more severe immune reaction.^{24,25} It could also be that staphylococcal superantigens or toxins would modify or exacerbate the immune response.²⁶

Multiple studies²⁷⁻³² have described mite cross-sensitization in dogs. Because the antibodies described in this study could target proteins that are present in other mites, we aimed to compare the same-pooled sera from these groups of dogs with another source of mite proteins. A western blot using a commercial extract of *D. farinae* was performed. Although one group of pooled sera (Fig. 2, lane 11) presented a band of reaction around the 10 kDa, the rest of the bands consistently showed a different pattern of reaction when compared to the western blot bands performed with the *D. canis extract* (Fig. 1).

The fact that in vitro culture systems for *Demodex spp*. have not been developed, the lack of availability of *Demodex* antigens represents a major handicap for the advancement of the immunological investigations in demodicosis. This work demonstrates that it is possible to obtain reasonable

quantity of purified *Demodex* extract to be used in immunological studies. Nevertheless, the method is very labour-intensive, and demands the manual collection of high number of mites. In our case, even after collecting 1200 mites, we needed to pool the sera. This is clearly one of the main limitations of this work. Another weakness of this study is that we could only investigate the presence of canine IgGs. It could well be that other immunoglobulin classes (IgA, IgE, IgM) against *Demodex* antigens are produced in healthy dogs or in dogs with generalized demodicosis. This point clearly deserves a future investigation. Furthermore, the availability of a good amount of *Demodex* extract would allow the characterization of the peptides and proteins of *Demodex*, maybe using MALDI-TOF or other highly sensitive techniques.

In short, this study reports that healthy dogs and dogs with CanJGD have serum antibodies directed against several *Demodex* antigens. This finding should prompt a more detailed investigation of the humoral immune response against *Demodex* mites and its role in health and in disease.

Aknowledgments

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10. DISCUSSION

Demodicosis is one of the most studied parasitic skin disease of humans and dogs, because it represents a unique disease in the context of its etiologic agent, and because of the many aspects of demodicosis that still are unknown. Demodicosis can be defined as an inflammatory skin disease characterized by the presence of *Demodex* mite overpopulation. It is almost "traditionally" explain in veterinary schools that in dogs, *Demodex* mites, are unequivocally present in the skin, and that dogs "inherit" the mites upon the first days of life. These concepts (almost axioms) that were scarcely investigated and challenged, probably were based on the earliest studies of CanD.¹ One of the purposes of our work was to challenge the concept of *Demodex* as a normal inhabitant of all dogs. Therefore, we developed a sensitive molecular technique to investigate our specific objectives.

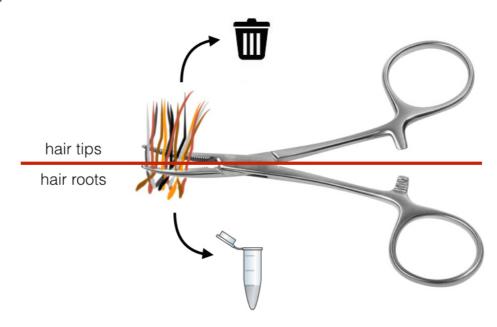
- "Can we use PCR technique?
- Well... sure. What kind of sample do you have?
- Canine hair samples!"

Since its origin in the early 1980s, PCR has become one of the most widely used tools in molecular biology.² Its capacity to generate results even starting with a small copy numbers of DNA material, converts PCR into one of the most sensitive techniques. But, how one can adapt such an exquisite technique to such a non-conventional sample as canine hairs? Although, previous efforts to detect *Demodex* DNA were made, our work was the first to perform a real-time PCR technique to detect Demodex DNA and to use this technique in canine hair samples and paraffin-embedded skin samples. In 2009, Borgo et al.³ used designed primers for the same chitin synthase (CS) gene as study 1 and a conventional PCR technique to amplify *Demodex* DNA from deep skin scrapings from twelve different dogs diagnosed with demodicosis. In this work, nine of twelve samples were positive in the PCR. In contrast, our technique amplified Demodex DNA in all (14/14) samples from canine demodicosis. One year later, Toops et al.4 could not amplify Demodex DNA using primers designed from DNA sequences obtained by a random amplified polymorphic DNA (RAPD) approach, nor by using primers designed from the same sequence of chitin CS from the Genbank that we used. As it was suggested in study 1, both the design of the primers and the use of a real-time PCR technique could explain our success compared with these previous attempts.

Sensitivity of hair plucking technique to detect *Demodex spp*. in healthy dogs is unknown, although sensitivity for the diagnosis of canine generalized demodicosis was reported to be of 97,3%.⁵ Hair plucking is a non-traumatic, fast, cheap, and very reproducible technique. In this way, we decided to use hair plucking technique to include only hair roots after hair plucking because if *Demodex* mites were present they would be attached to the hair roots. Briefly, after selecting the dog's location (*i.e.* interdigital skin of the feet, periocular, dorsum, etc.), hair plucking was performed with a hemostatic clamp in a way that hair roots were at one side of the clamp and the hair tips at the other, so

they can be discarded to only include hair roots (Figure 4). Hair roots were included in an eppendorf tube containing saline solution, labeled with the dog's identification and sample location, and freeze at -20°C until DNA extraction was performed.

Figure 4. Hair plucking technique. Note that only hair roots were included in the eppendorf tube.



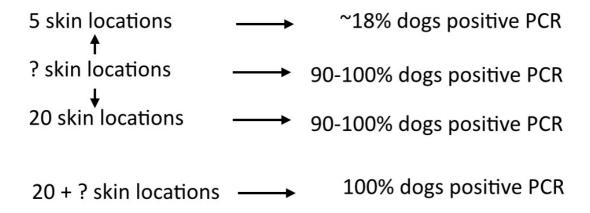
- "I didn't see mites under the microscope, how the PCR could be positive?"

When we were setting-up the PCR technique, in addition to the positive controls (dogs with demodicosis), many samples were observed under the light microscope with the aim of comparing molecular data with morphological observation of mites. In many of the cases assessed in parallel, *Demodex* mites were not seen under the microscope, but resulted positive by real-time PCR. This can be explained by the fact that visualize *Demodex* mites in such an extensive amount of hair roots can be very difficult and laborious. Unfortunately, due to the DNA extraction procedure, those samples were not able to being observed under the microscope for a second time. Another possible explanation of this disagreement, would be the presence of *Demodex* mite fragments in the skin scraping samples, though positive in the real-time PCR, unnoticed in the microscope observation. Also, Demodex feces would be also positive in the real-time PCR technique, although unobservable in the light microscope. The results demonstrated that real-time PCR technique (study 1), is a highly sensitive diagnostic technique, useful and low time consuming for the clinical and epidemiological settings (study 3).

- "Does all dogs harbor Demodex mites on their skin?"

Results from study 1 and study 3 brought new insights to the traditional concept of *Demodex* mites being permanent inhabitant of the canine skin. Real-time PCR amplifying a 166-bp fragment of the CS gene showed that when sampling five different skin locations, the percentage of dogs harboring *Demodex* mites were around 18%. However, if the number of skin locations is increased to twenty (like in experiments 3 and 4 of study 3) the percentage of dogs harboring *Demodex* mites would range between 90% and 100%. Taking into account these results, the next questions would be "which is the minimum number of skin locations necessary to reach 100% of positive dogs and which is the limiting number of skin locations that would reach between 90 and 100%?" This is explained in figure 5.

Figure 5. How many skin locations (between 5 and 20) are necessary to reach 90-100% of positive dogs? How many skin locations are necessary to reach 100% of positive dogs?



Unfortunately, the answers to these questions are unknown, but these results give us an approximation. Based on these experiments (study 3), we can not conclude that all dogs harbors *Demodex* mites on their skin, but we were able to amplify *D. canis* DNA from the skin of most healthy dogs. Therefore, *Demodex* can be considered a normal inhabitant of the canine skin, however, the population of mites in a healthy dog must be very small because we had to increase the number of sampling sites to be able to detect the parasite in a majority of dogs.

- "Is Demodex like a rolling stone?"

Once demonstrated that *Demodex* mites are normal inhabitants of the canine skin, our interest was to determine the distribution of the *Demodex* mites on the canine skin. In human beings, *Demodex folliculorum* are distributed in a regionalized form, being the face and head the most populated regions⁶ and also having a few small ectopic niches such as the nipples, the hairy chest, and the genital area.^{7,8} On the other hand, *D. brevis* seems to have a wider distribution in the human skin.⁹ However, some authors have suggested that

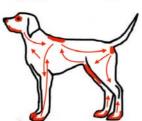
human *Demodex* mites are present in all parts of the skin of the human body. 10,11

Based on our results, a generalized pattern of distribution for *D. canis* seems unlikely because of the following reasons: (1) some dogs consistently gave a negative PCR result (study 3, experiment 2: 25 of 30 dogs negative in experiment 1 were still negative 6 months later); (2) even the most generalized clinical cases of CanD can spare a few areas such as the tail; and (3) lesions of clinical cases of both localized or generalized CanD, usually starts in confined areas (periocular, perioral, feet, among others), instead of a random configuration. According to our data, defined spots of small Demodex mite populations seem more likely in canine skin. Spots of small Demodex mite populations would explain why some dogs had consistently negative or positive PCR results in certain skin locations. Moreover, spots of *Demodex* populations could be static (no marked changes in Demodex density through time) or dynamic (continuous Demodex demographic fluctuations). The idea of a spotted pattern of distribution with Demodex mites repopulating the spots by means of migration movements would explain why through time (6 months) a PCR result may turn from negative to positive and vice versa. Results of the experiment 2 of study 3 support this later hypothesis. However, *Demodex* DNA was amplified from all the cutaneous locations investigated, without statistically significant differences. The generalized pattern or the spotted pattern of distribution with Demodex showing nomadic behavior would explain why all cutaneous locations tested positive for *Demodex DNA*. Although speculative, possible patterns of canine Demodex population distribution are depicted in figure 6.

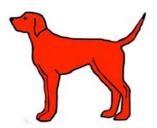
Figure 6. Suggested possible patterns of *Demodex* spp. population distribution.



A. Regional pattern: one or a few regions represent the heavy density population with or without small ectopic niches that are repopulated by the predominant regions.



- B. Spotted pattern: in terms of *Demodex* density:
- (a) static spots,
- (b) dynamic spots.



C. Generalized pattern: Demodex mites all present all over the skin.

In contrast to *D. canis*, *D. injai*, inhabits deeper parts of hair follicles, sebaceous glands and sebaceous ducts of the canine skin. Most clinical reports confined CanD due to *D. injai* to a very specific cutaneous area, the dorsal midline. Since no study has been done regarding *D. injai* populations, almost nothing is known about *D. injai* prevalence and anatomic distribution in healthy dogs. Although, clinical cases have associated *D. cornei* with *D. canis* overgrowth, nothing is known about *D. cornei* prevalence and anatomic distribution. Taking into account the results from case reports, ¹⁵⁻²⁰ *D. cornei*, possibly may have a similar pattern distribution as *D. canis*; although, this remains under the speculative field.

- "Phenotype versus genotype. One for all or different species?"

In 1942, Mayr defined what is known as the biological species concept, which stated that species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups.²¹ Two populations are not part of the same species if their constituent organisms are reproductively incompatible in sympatry (geographical overlapping populations). However, reproductive compatibility is not an accurate measure and it not takes into account the underlying process of evolution.²² Almost forty vears later. Wiley revised what is it known as the evolutionary species concept that took into account the recovered history of evolution of a particular species. According to Wiley, 23 species can be defined as a single lineage of ancestraldescendant populations which maintains its identity from other such lineages, has its own evolutionary tendencies and historical fate, and morphological distinctiveness is not necessary. Finally, in 1983 Cracraft proposed what is known as the phylogenetic species concept. This new concept defined species as the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent.²⁴ Accordingly, none of these concepts represents the absolute true but, different interpretations according to different times.

In the past, *Demodex* species differentiation was based on morphological features (similarities and differences in their phenotypic characteristics) and host identification. The advent of molecular biology resolved many problems intractable for morphologists; providing a phylogenetic record from very recent time to the origin of life on Earth. One of the objective of phylogenetic studies is to reconstruct the evolutionary history of a group of organisms.²⁵ The invention of PCR and automated DNA sequencing almost three decades ago, allowed the comparison of these sequences within living organisms.²⁶ Mitochondrial DNA (mtDNA) sequences are among the most rapidly evolving DNA sequences of eukaryotes,²⁵ and this results in the accumulation of differences between closely related species.²⁷ Furthermore, mtDNA is useful for phylogenetic studies because of their matrilineal inheritance, lack of extensive recombination, and accelerated nucleotide substitution rates.²⁸ Although, our real-time PCR technique from study 1 was not designed for species identification purposes, it yielded an interesting result. The amplification product (166-base pair) of *D. injai* showed changes in seven nucleotides in contrast to D. canis and the sequence of this fragment was identical between D. canis and the unnamed short-bodied

Demodex (*D. cornei*). This leaded us to the suspicion that a phylogenetic comparison of the species of canine *Demodex* mites could be investigated (study 2). To compare phylogeny of canine *Demodex* mites, we used similar primers of a previous study²⁹ to amplify a 338bp DNA fragment of the mitochondrial 16S ribosomal DNA (rDNA). In our study, for the comparison and phylogenetic analysis, a fragment of 228bp that included the region of the highest variability was used.

In addition to its morphological differences, cutaneous habitat, and distinct clinical presentation, study 2 suggested that *D. injai* might be a different species from *D. canis*. The sequences of the two isolates of *D. injai* besides from being identical, they showed 76.6% identity with the *D. canis* sequence. Taking into account the percentage of similarity to *D. canis* sequence (study 2 isolates and two published GenBank sequences), it can be said that *D. folliculorum* is more similar to *D. canis* than *D. injai*. On the other hand, the short-bodied *D. cornei* showed 98.2% and 99.6% sequence similarity to the two GenBank sequences, and 97.8% to our *D. canis* isolates (table 7). This suggests that *D. cornei* may only differ in terms of morphology (a different phenotype) from *D. canis*. This would be in agreement with one study which proposed that morphology of canine *Demodex* mites varied depending on the condition studied (breed, host response, body site, surface or depth), suggesting an unique species with different morphological dimensions.³⁰

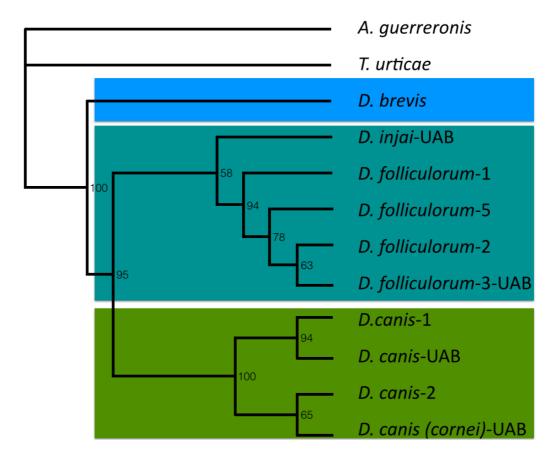
Table 7. Results from study 2. H, number of haplotypes; Hd, haplotype; π , nucleotide diversity; N/E, not estimated. *Similarity to two *D. canis* sequences from GenBank.

	Sequence analysis			Divergence
	Н	Hd	π	Similarity to <i>D. canis</i> isolates sequence (%)
D. canis	4	0.750	0.014	99.6 and 97.3*
D. cornei	N/E	N/E	N/E	97.8
D. injai	1	N/E	N/E	76.6
D. folliculorum	4	0.643	0.011	80.1
D. brevis	1	N/E	N/E	N/E

An evolutionary tree (phylogenetic tree or phylogeny) is a diagrammatic depiction of biological entities that are connected through common descent, such as species or higher-level taxonomic groupings.31 As it name defines it, it is composed of a root, branches, nodes, and tips or leaves. The root represents the last common ancestor. The only way to root a tree is with an out-group, an external point of reference that is not a natural member of the group of interest. In our case, two out-groups were selected: Aceria guerreronis (coconut mite) and Tetranychus urticae (red spider mite of plants). Branches connect nodes; a node is the point at which two (or more) branches diverge.³² Nodes represent the common ancestors from which two or more related lineages are descended, and the tips are individual species or larger taxonomic groups, being contemporaries of one another. 31 The lengths of the branches correspond to the amount of evolution (roughly, percent sequence difference) between the two nodes they connect. The analysis of the phylogenetic tree of *Demodex* species (Figure 7) from study 2, clearly shows three different branches: (1) D. brevis, (2) D. injai and D. folliculorum, and (3) D. canis. Bootstraping is a common

statistical method for assessing confidence in phylogenetic analysis.³³ This is done by taking random subsamples of the dataset (in our case, the *Demodex* sequences), building trees from each of these subsamples and calculating the frequency with which the various parts of the tree are reproduced in each of these random subsamples. Each of the subsamples are the same size as the original, and this is accomplished by random sampling with replacement.³² When a bootstrap is 100%, it means that that particular node (group) appears in every subsample. According to Hillis,³³ bootstraps proportions of \geq 70% correspond to a probability of \geq 95% that the corresponding clade is real. In our case, the three different branches had a bootstrap proportion of 100% and 95%.

Figure 7. Phylogenetic tree of *Demodex* species inferred from partial sequences of mitochondrial 16S rDNA showing 3 distinct branches.



In 2003, Hebert introduced the term "DNA barcode". Barcoding provides a standardized method by the use of a short DNA sequence from a particular region of the genome to provide a 'barcode' for identifying species. This fragment of the mtDNA gene (approximately 600-base pair) codes for a subunit of the enzyme cytochrome oxidase I (COI) and also served for the identification of many animal species by defining a range of intraspecies identification standards: divergences, no greater than 2 %; genetic distance, less than 0.020. One year later, the same author, extended earlier investigations and proposed a standard screening threshold of sequence difference, which could speed the discovery of new animal species. This threshold stated that species

differentiation are possible when the interspecific difference is about ten times the average of the intraspecific difference of the group studied.²⁷ In study 2, the mean interspecific distance was approximately 14.5 times greater than the intraspecific distance found in *D. canis*, which supports the idea of *D. injai* as a different species from *D. canis*.

Since the publication in 2011 of our real-time PCR technique (study 1), many studies have been carried out and new findings were reported in relation to *Demodex* species. In the same year, de Rojas et al.³⁵ compared two populations of *D. folliculorum* from humans isolated from different habitats (skin and eyelashes follicles). A 436-bp region of the 16S rDNA and a 453-bp region of the COI gene from individual mites of each population were sequenced. Morphological and biometrical differences were detected between *D. folliculorum* from human eyelashes and skin. On the other hand, based on COI gene sequences, endonucleases that could be used to delineate both populations of *D. folliculorum* were identified. However, based on 16S rDNA analysis, no significant intraindividual polymorphisms were detected, suggesting that *D. folliculorum* subspecies were unlikely.

Later, Zhao and Wu³⁶ applied sequence characterized amplified regions (SCAR) based on the RAPD to discriminate between 6 isolates of three species of Demodex (D. canis, D. brevis, and D. folliculorum), Briefly, this technique is based on the evaluation of different primers until one or several are found to produce specific DNA bands that can act as universal markers for a given species. In this study, 10 primers were tested and it was found that one of the designed primers could be used to identify and classify the three species under study. Another important finding showed that the interspecies genetic distances between D. folliculorum and D. canis were shorter than that between D. folliculorum and D. brevis. One year later, the same authors, 29 reported phylogenetic relationships in *Demodex* mites based on mitochondrial 16S rDNA partial sequences. In addition to confirm previous results, they showed that mitochondrial 16S rDNA partial sequence could identify the difference among D. canis, D. brevis, and D. folliculorum species. On the other hand, the phylogenetic trees did not reveal subspecies differentiation of two geographic D. folliculorum isolates (China and Spain).

In 2012, de Rojas et al.³⁷ reported its results by using amplification and sequencing of the 16S rDNA and for the first time, COI mitochondrial genes of *D. canis*, *D. cornei*, and *D. injai*. The authors found unexpected low values of divergence and genetic distance between the three canine *Demodex* species. Moreover, phylogenetic tree based on COI sequence gathered together *D. cornei* and *D. injai*, separated from *D. canis*. Based on these results, the authors suggested that *D. canis*, *D. injai*, and *D. cornei* are polymorphism of the same species. This is in contrast with our previous results (study 2), and the reasons of these differences could be explained by the use of a different DNA extraction method, different primers (not from *Demodex* sequences), or misjudged morphological *Demodex* classification. In addition, another study³⁸ confirmed *D. injai* as a separate species from *D. canis*, suggesting that the small sample numbers could explain the difference found in 16S rDNA sequence (study 2) and COI sequence.^{37,39}

Another study from Zhao et al.⁴⁰ challenged two geographical isolates of *D. canis*, this time, from China and Japan. By cloning and sequencing the CS gene (same as study 1), they found that sequence similarities between three Chinese *D. canis* isolates and one Japanese *D. canis* isolate ranged from 99.7% to 100.0%, and those between four *D. canis* isolates and one *D. brevis* isolate were 99.1%-99.4%. Furthermore, the authors deduced that the CS gene resulted to be a class A gene, which is associated with chitin synthesis in the integument of *Demodex* mites.

In 2014, a study from Zhao et al.⁴¹ proposed that a 429-bp mitochondrial COI fragment could be an ideal DNA barcode for molecular classification, identification, and phylogenetic study of *Demodex* species. Once again, in this latter study, it was demonstrated that *D. folliculorum* is closer to *D. canis* than to *D. brevis*.

- "Demodex injai has its own"

To further support our previous findings in study 2, a conventional PCR technique to specifically amplify 16S rDNA from *D. injai* was designed (study 4). Two important conclusions can be retrieved from this study. First, that *D. injai* could be part of the normal fauna of some dogs, since two of nineteen healthy dogs were positive for the specific *D. injai* PCR. Second, *D. injai* overgrowth seems to be uncommon in dogs with juvenile generalized demodicosis, since in the seven cases of juvenile generalized demodicosis investigated, mite overgrowth did not included *D. injai* overgrowth. The low number of cases and *D. injai* isolates used for DNA amplification represents one limitation of this study. Furthermore, only one positive control and three dogs with seborrhea associated with *D. injai* overgrowth were included in this study. However, positive controls of study 4 were made with samples of *D. injai*, *D. canis*, and *D. folliculorum* mites with each tube containing one mite each. This demonstrates the high sensitivity of the specific *D. injai* PCR technique, since it only amplified DNA in the *D. injai* containing tube.

In 2013, Milosevic et al.⁴² reported a new localization of *D. injai* based on a case report of a 12-year-old spayed female beagle dog with a bilateral ceruminous otitis externa. By amplification and sequence of 16S rDNA, the authors confirmed *D. injai* as a different species from *D. canis*. Although, different primers were used, this study supported our findings of study 2 and 4 where *D. injai* is proposed as a different species, and of study 2 regarding the sequence similarities between *D. injai* and *D. folliculorum*. In addition, while studying mitochondrial 16S rDNA fragments of *Demodex caprae*, Zhao et al.³⁸ also proposed *D. injai* as a separate species from *D. canis*.

- "Some concluding remarks on phylogeny"

From the studies above, a few conclusions can be retrieved. First, *Demodex* mites from a same species are similar regardless of geographical location. In other words, *D. folliculorum* mites are similar regardless if the

samples are taken from China or from Spain.^{29,43} Similarly, *D. canis* mites are similar regardless if the samples are taken from China or from Japan.⁴⁰ Second, *D. injai* seems definitively a different canine *Demodex* species. This is supported by the fact that *D. injai* has a distinct skin habitat (sebaceous gland and sebaceous ducts); when produce demodicosis, *D. injai* has a distinct clinical presentation, and it has a sufficient interspecific difference compared to *D. canis*.^{38,42,43} Finally, from a phylogenetically point of view, evidence suggest that *D. folliculorum* is closer to *D. canis* than *D. brevis*, which suggest a shared common ancestor and shows the tight relationship between the men and dog since domestication of the former.

- "The humoral response against Demodex canis"

It is assumed that dogs with generalized demodicosis have an adequate humoral immune response. This statement is based on some of the studies showed in table 6 (Introduction section, 3. 5. Immunology of canine demodicosis). Although previous studies^{44,45} reported the use of *Demodex* proteins to investigate the role of the host immune system in the pathogenesis of demodicosis, study 5 is the first study that describe the use of *D. canis* proteins to detect anti-mite canine IgG antibodies by western blot in a group of healthy dogs and in groups of dogs with CanD with or without secondary pyoderma. The analysis of the western blot using a crude *D. canis* protein extract revealed that both healthy dogs and dogs with CanD showed IgGs against *D. canis* antigens. Three distinct responses were observed:

- (1) The healthy dogs: when pooled sera from a group of healthy dogs (n = 19) with no history of CanD was incubated, bands of reaction were observed against 55-kDa to 72-kDa proteins of *D. canis*. This means that there was a humoral immune response against *Demodex* mites in these healthy dogs, although the function and importance of this response remains unknown. One explanation could be that this response is part of a mechanism to control mite populations, as it was suggested in an experimental model. The anti-*Demodex* IgGs could trigger some effector mechanisms to prevent mite overgrowth. However, they could also be irrelevant, an epiphenomenon consequence of the presence of the mites in the hair follicles. A balanced relationship between the host's immune system and the presence of the mites is proposed, since *D. canis* is considered a normal inhabitant of the dog's hair follicle. In this way, a constant level of exposure to the mite antigens would be present, which could generate a humoral response against specific regularly exposed proteins, without a physiological role.
- (2) Dogs with CanD with secondary pyoderma: pooled sera from this dogs (n = 17) reacted mainly against a 10-kDa protein. Is it known that during disease, following mite overpopulation, enlargement and inflammation of the hair follicle occur^{47,48} producing the typical appearance of moth-eaten alopecia, erythema, papules, and scaling.⁴⁸⁻⁵¹ Mite secretions, excretions, and somatic debris accompany multiplication of mites inside the hair follicles.^{48,49} By seeping out of the hair follicle (follicular rupture), these foreign material from *Demodex* origin may constitute a possible source of antigen(s) to which the host is apt to

react.⁴⁸ Moreover, mites and hairs released into the dermis give access to bacteria normally found on dog's skin resulting in a local pustular reaction, which may become extensive giving appearance to secondary pyoderma.⁵² This pustular form is usually a severe disease which can terminate fatally if not treated.^{50,53}

(3) Dogs with CanD without secondary pyoderma: sera from these dogs (n = 14) reacted against diverse antigens, 55, 72, and 10-kDa. This suggests an overlapping response between the two formers. It could be that dogs with a mild to moderate mite overgrowth had a humoral response that approaches that of the healthy dogs. When mite overgrowth progress to considerable numbers but without clinically manifesting secondary pyoderma, humoral response may switch to the 10-kDa target.

Results from works that studied CanD skin lesions seemed to be in agreement with this explanation. In one study, the inflammatory patterns and immunophenotype of the histologic lesions of CanD were described. The lesions of furunculosis (late stage of hair follicle inflammation) contained greater numbers of IgG-secreting plasma cells and fewer T lymphocytes than did the cases with perifolliculitis (early stage of hair follicle inflammation). The authors of this study suggested that this might represent a shift from Th1 (cell-mediated) immune response toward Th2 (humoral) response in cases of furunculosis, were is present an extra antigenic stimulus. In a similar study, plasma cells expressing cytoplasmic IgG were more numerous than those bearing IgM or IgA. Furthermore, the authors of this study observed that IgG response subclass in lesions of perifolliculitis to furunculosis became polyclonal in nature, with mixed IgG2 and IgG4 expression, and that such a progression may be driven by sequential exposure to different *Demodex* antigens.

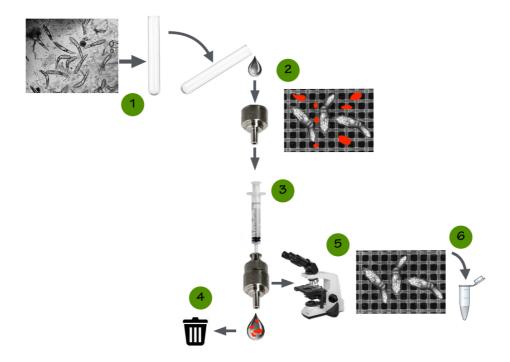
Multiple studies⁵⁶⁻⁶¹ described mite cross-sensitization in dogs. The antibodies described in study 5 by the western blot technique with the *D. canis* extract may target proteins that are present in several mites. In order to compare the same pooled sera from these groups of dogs with other source of proteins, a western blot using a commercial extract of *Dermatophagoides farinae* was performed. Although one group of pooled sera (fourth group of pooled sera from dogs with CanD with secondary pyoderma) presented a band of reaction around the 10-kDa, the rest of the bands showed a different pattern when compared to the western blot bands performed with the *D. canis* extract. According to one study⁶² using a lyophilized whole-body *D. farinae* crude extract at the same concentration as study 5, the most commonly recognized bands in healthy and atopic dogs were 18-kDa and 98-kDa antigens for IgG1, and 18, 45, 66, 98, 130, and 180-kDa for IgG4. Bands of reaction at 10-kDa were not observed.

Some proteins of the mite *Sarcoptes scabiei* cross-react with other mite proteins. ^{56,63,64} As a limitation, study 5 did not include a third western blot using *S. scabiei* antigens in order to compare it with the *D. canis* western blot. In one study, ⁶⁵ a western blot performed with a crude *S. scabiei* extract, and with pooled sera from dogs with sarcoptic mange showed predominant bands of reaction of 164-kDa and 147-kDa. The sera of dogs without sarcoptic mange

did not reveal any specific antibody reaction when probed in the western blot. In addition, sera from dogs with confirmed infections with *Cheyletiella sp.* (skin mite), *D. canis*, *Linognathus setosus* (dog's louse) and *Otodectes cynotis* (ear mite), and sera from dogs diagnosed as being allergic to fleas were tested by enzyme-linked immunosorbent assay using the same *S. scabiei* extract. All sera had optical density values below 0,15 (negative).

Another limitation of study 5 would be the use of pooled sera instead of individually sera. The method of collection of *Demodex* mites performed in this study, although simple, was effective at the time of acquiring entire-body mites without epithelial cells or debris, and without damaging consequences for the mite structure. However, this method is a most time consuming procedure, and renders low protein levels. In our case, around 1.200 *D. canis* mites rendered a mean protein concentration of 6,8 ng/µL. In this way, we consider that the use of pooled sera was our best option in terms of detecting bands of reaction in the western blot. Nevertheless, we can define this method as, simple, inexpensive, and reproducible (figure 8).

Figure 8. This figure shows the method of collection of *Demodex canis* for protein extraction.



- (1) Skin scrapings containig *Demodex canis* mites were transferred into a glass tube containing PBS.
- (2) The mixture was placed on an 11µm nylon net filter held in a stainless steel filter holder.
- (3) The nylon net filter was washed three times with PBS and filtered.
- (4) The filtered liquid was discarded.
- (5) The nylon net filter was placed on a glass slide and visualized under the microscope.
- (6) Each mite was retrieved from the nylon net filter and placed into an eppendorf tube with PBS.

In 2007, a gram-negative bacteria (*Bacillus oleronius*) was isolated from a *D. folliculorum* mite extracted from the face of a patient with papulopustular rosacea.⁶⁶ Western blot analysis revealed the presence of two antigenic

bacterium proteins (62 and 83 kDa) when incubated with sera from patients with rosacea (controls were negative). The 62-kDa protein shared amino acid sequence homology with an enzyme involved in carbohydrate metabolism and signal transduction, while the 83-kDa protein was similar to bacterial heat shock proteins. The authors of this study suggested that the presence of multiple mites in individual follicles could distend or damage the follicular integrity to an extent that allows diffusion of these mite-related bacterial antigens through the follicular wall, triggering a perifollicular host immune response. In another study, 67 sera from patients with erythematotelangiectatic rosacea also reacted with the 63 and/or 82-kDa protein(s) of *B. oleronius*. Moreover, these patients displayed a higher population of *Demodex* mites in their skin and a lower level of sebum than controls. On the other hand, a small proportion (30%) of sera from healthy controls also reacted with the 63 and/or 82-kDa protein(s) of *B. oleronius*.

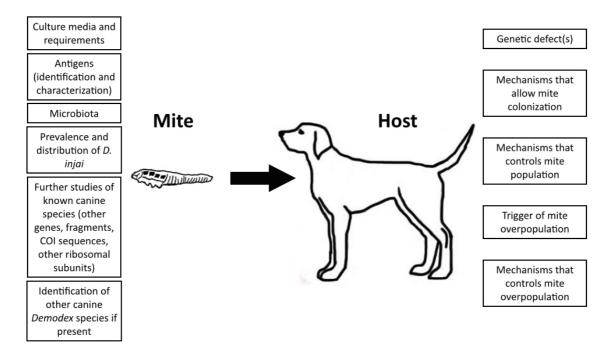
In the case of dogs, the presence of bacteria such as *B. oleronius* in *D. folliculorum*, has not yet been studied. Some of our results from study 5 may also reflect the immunoreaction between the studied pooled sera and antigens from an unknown endobacteria of *D. canis*. However, this hypothesis would not explain the differences in molecular weights immunoreactivity between healthy dogs and dogs with demodicosis. Interestingly, *Demodex* microbiota from rosacea patients and controls was recently reported by Murillo et al.⁶⁸ A total of 86 species were identified with 36 as *Demodex*-specific microbiota. The authors found an unsuspected diversity of the microbiota of human *Demodex*, which seemed to vary according to host status (papulopustular rosacea *versus* erythematotelangiectatic rosacea *versus* healthy controls). Interestingly, they were unable to identify *B. oleronius*.

- "Looking to the future".

Since it is reasonable to think that hundreds of *Demodex* species colonize the skin of mammals, it seems probable that future investigations will extend our knowledge in phylogenetic relationships between other *Demodex* mites from different species. Although very recently, phylogenetic studies has been done on *Demodex* from cats^{69,70} and goats,^{38,41} there is still a paucity of studies in other mammals. For example, *Demodex* mites have not yet been retrieved from wolfs. Since dogs probably have an East Asian origin from wolves approximately 15.000 years ago,⁷¹ *Demodex* mites from wolves (if present) would give us information about ancestors of *Demodex* mites in dogs.

Demodex mites, and in particular, demodicosis in dogs, have currently large uncovered areas of study. Figure 9 depicts the main topics that we believe that demand urgent consideration for research. Unquestionably, those tasks are extremely difficult and even in human dermatology are still unresolved. Unmasking some of the points considered in figure 9 will allow a better understanding of disease predisposition, progression, and prevention. We consider that the use of a *Demodex canis* antigen extract presented in this doctoral thesis represents a crucial step for future research in the field of dog's immune response against *Demodex* mites and demodicosis.

Figure 9. Main topics of *Demodex* and CanD that requires further investigation.



11. CONCLUSIONS

Based on the results of the five different studies presented here, we can conclude the following:

- (1) A real-time PCR technique that amplifies a fragment of the chitin synthase gene has demonstrate to be a specific and sensitive technique to detect *Demodex* DNA in different canine samples. The technique has proven to be extremely useful tool for performing epidemiologic studies.
- (2) The real-time PCR technique was able to detect *Demodex* DNA in the skin of all healthy dogs investigated, and therefore *Demodex* mites have to be considered normal inhabitants of the canine skin. They are probably distributed in very low numbers along all the haired skin.
- (3) Amplification and sequencing of a fragment of the mitochondrial 16S rDNA gene showed that *D. injai* is a different species from *D. canis*, and that *D. cornei* is probably a morphological variant of *D. canis*.
- (4) Healthy dogs and dogs with canine juvenile generalized demodicosis have a specific acquired humoral immune response against *Demodex* and present serum antibodies directed against several *Demodex canis* protein antigens.

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13. ANNEX

	Author	Year	Publication	Source	Species retrieved
folliculorum	Simon G	1842	Ueber eine in den kranken und normalen Haarsacken des Menschen lebende Milbe	Archiv für Anatomie, Physiologie und Wissenschaftliche Medecin	Human (Homo sapiens)
canis	Leydig F	1859	Ueber Haarsackmilben und Krätzmilben	Archiv für Naturgeschichte	Dog (Canis familiaris)
phyllostomatis	Leydig F	1859	Ueber Haarsackmilben und Krätzmilben	Archiv für Naturgeschichte	Surinam bats (Phyllostoma hastatum)
arvicolae	Zschokke S	1888	-	Archiv für Tierheilkunde	Field vole (<i>Microtus agrestis</i>)
cati	Megnin JP	1877	Memoire sur le <i>Demodex folliculorum</i> Owen	Journal of Anatomy and Physiology	Cat (Felis domesticus)
phylloides (syn.: suis)	Csokor J	1879	Ueber Haarsackmilben und eine neue Varietät derselben bei Schweinen, <i>Demodex phylloides</i>	Verhandlungen der Zoologisch- Botanischen Gesellschaft in Wien	Pig (Suis scrofa)
bovis	Stiles CW	1892	On Demodex folliculorum var. bovis in american cattle	The Canadian Entomologist	Cattle (Bos taurus)
equi	Railliet A	1895	-	Traité de zoologie médicale et agricole	Horse (<i>Equus caballus</i>)
caballi	Railliet A	1895	-	Traité de zoologie médicale et agricole	Horse (<i>Equus caballus</i>)
ovis	Railliet A	1895	-	Traité de zoologie médicale et agricole	Sheep (Ovis aries)

Species	Author	Year	Publication	Source	Species retrieved
caprae	Railliet A	1895	-	Traité de zoologie médicale et agricole	Goat (Capra hircus)
musculi	Oudemans A	1897	List of Dutch <i>Acari,</i> 7 part: <i>Acaridiae</i> Latr., 1896, and <i>Phytoptidae pagenst.</i> , 1861, with synonymical remarks in description of new species	Tijdschrift voor Entomologie	House mouse (Mus musculus)
cuniculi	Pfeiffer	1903	Acarus folliculorum cuniculi	Berlin Tierärztliche Wochenschrift	European rabbit (Oryctolagus cuniculus)
muscardini	Hirst S	1917	On three new parasitic <i>Acari</i>	Annals and Magazine of Natural History	Dormouse (Muscardinus avellanarius)
erinacei	Hirst S	1917	On three new parasitic <i>Acari</i>	Annals and Magazine of Natural History	Hedgehog (<i>Erinaceus europaeus</i>)
ratti	Hirst S	1917	Studies on Acari: No. 1. The genus Demodex, Owen	Brittish Musseum (Natural History)	Brown rat (Rattus norvegicus)
soricinus	Hirst S	1918	On four new species of the genus Demodex, Owen	Annals and Magazine of Natural History	Common shrew (Sorex araneus castaneus)
apodemi	Hirst S	1918	On four new species of the genus ${\it Demodex}$, Owen	Annals and Magazine of Natural History	Striped field mouse (Apodemus agrarius)
longior	Hirst S	1918	On four new species of the genus ${\it Demodex}$, Owen	Annals and Magazine of Natural History	Wood mouse (Apodemus sylvaticus)
nanus	Hirst S	1918	On four new species of the genus <i>Demodex</i> , Owen	Annals and Magazine of Natural History	Brown rat (<i>Rattus</i> norvegicus), and black rat (<i>Rattus rattus</i>)

Species	Author	Year	Publication	Source	Species retrieved
ermineae	Hirst S	1919	Studies on Acari: No. 1. The genus Demodex, Owen	Brittish Musseum (Natural History)	Stoat (<i>Mustela erminea</i>)
glareoli	Hirst S	1919	Studies on Acari: No. 1. The genus Demodex, Owen	Brittish Musseum (Natural History)	Bank vole (Myodes glareolus)
chiropteralis	Hirst S	1921	On some new little-known Acari, mostly parasitic in habit	Proceedings of the Zoological Society London	Long-eared bat (Flecotits auritus)
melesinus	Hirst S	1921	On three new Parasitic Mites (Leptus, Schöngastia, and Demodex)	Annals and Magazine of Natural History	Badger (<i>Meles taxus</i>)
gliricolens	Hirst S	1921	On some new or little-known Acari, mostly parasitic in habit	Proceedings of the Zoological Society London	Water rat (Arvicola amphihius)
talpae	Hirst S	1921	On some new or little-known Acari, mostly parasitic in habit	Proceedings of the Zoological Society London	Mole (<i>Talpa europaea</i>)
саvіае	Bacigalupo J	1954	Demodex caviae n. sp.	Revista de Medicina Veterinaria de Buenos Aires	Guinea pig (<i>Cavia porcellus</i>)
criceti	Nutting WB	1958	Demodex criceti n. sp. (Acarina: Demodicidae) with notes on its biology	The Journal of Parasitology	Golden hamster (Mesocricetus auratus)
aelleni	Fain A	1960	Les acariens psoriques parasites des chauves-souris. XIII. La famille <i>Demodicidae</i> Nicolet	Acarologia (Paris)	Daubenton's bat (Myotis daubentonii)
aurati	Nutting WB	1961	Demodex aurati sp. nov. and D. criceti, ectoparasites of the golden hamster (Mesocricetus auratus)	Parasitology	Golden hamster (Mesocricetus auratus)

Species	Author	Year	Publication	Source	Species retrieved
myotidis	DiBenedetto SA	1961	The biology of <i>Demodex myotidis sp. nov. (Acarina:</i> Demodicidae) from three species of bats	M. A. Thesis Univ. Massachusetts	Species of bats
sylvilagi	Maravelas GK	1962	Studies of Demodex sylvilagi n.sp. (Acarina: Demodicidae) from the New England cottontail Sylvilagus transitionalis bangs with a description of Demodes transitionalis n.sp.	M. A. Thesis Univ. Massachusetts	New England cottontail (Sylvilagus transitionalis)
brevis	Akbulatova L	1963	Demodicosis in man	Vestnik dermatologii i venerologii	Human (<i>Homo sapiens</i>)
antechini	Nutting WB	1970	Demodex antechini (Acari, Demodicidae) parasitic on Antechinus stuartii (Marsupialia)	Parasitology	Brown antechinus (Antechinus stuartii)
carolliae	Desch CE	1971	Parasitic mites of Surinam. I. <i>Demodex carolliae</i> sp.nov. (<i>Acari: Demodicida</i> e) from the fruit bat <i>Carollia perspicillata</i>	Parasitology	Fruit bat (Carollia perspicillata)
gapperi	Nutting WB	1971	Demodex gapperi sp.n. (Acari: Demodicidae) from the red- Backed vole, Clethrionomys gapperi	The Journal of Parasitology	Southern red-backed vole (Clethrionomys gapperi)
longissimus	Desch CE	1972	Parasitic mites of Surinam VII: Demodex longissimus n.sp. from Carollia perspicillata and D. molossi n.sp. from Molossus molossus (Demodicidae: Trombidiformes); Meibomian complex inhabitants of neotropical bats (Chiroptera)	Acarologia (Paris)	Seba's short-tailed bat (Carollia perspicillata)
molossi	Desch CE	1972	Parasitic mites of Surinam VII: Demodex longissimus n.sp. from Carollia perspicillata and D. molossi n.sp. from Molossus molossus (Demodicidae: Trombidiformes); Meibomian complex inhabitants of neotropical bats (Chiroptera)	Acarologia (Paris)	Velvety free-tailed bat (Molossus molossus)
melanopteri	Lukoschus FS	1972	Parasitic mites of Surinam XII. Demodex melanopteri sp.n. (Demodicidae: Trombidiformes) from the Meibomian glands of the neotropical bat Eptesicus melanopterus	Acarologia (Paris)	Brazilian brown bat (<i>Eptesicus melanopterus</i>)
saimiri	Lebel RR	1973	Demodectic mites of subhuman primates. I. Demodex saimiri sp.n. (Acari: Demodicidae) from the squirrel monkey, Saimiri sciureus	The Journal of Parasitology	Common squirrel monkey (Saimiri sciureus)

Species	Author	Year	Publication	Source	Species retrieved
lacrimalis	Lukoschus FS	1974	Demodex lacrimalis spec.nov. (Demodicidae: Trombidiformes) from the Meibomian glands of the European wood mouse Apodemus sylvaticus	Acarologia (Paris)	Wood mouse (Apodemus sylvaticus)
odocoilei	Desch CE	1974	Demodex odocoilei sp.nov. from the white-tailed deer, Odocoileus virginianus	Canadian Journal of Zoology	White-tailed deer (Odocoileus virginianus)
ghanensis	Oppong ENW	1975	Demodex ghanensis sp.nov. (Acari, Demodicidae) parasitic on west African cattle	Ghana Journal of Science	Cattle (Bos taurus)
cafferi	Nutting WB	1979	Demodex cafferi n.sp. from the African buffalo Syncerus caffer	International Journal of Acarology	African buffalo (S <i>y</i> ncerus caffer)
marsupiali	Nutting WB	1980	Parasitic mites of Surinam XXXVII. Demodex marsupiali sp.nov. from Didelphis marsupialis: adaption to glandular habitat	Zoologische Mededelingen	Common opossum (<i>Didelphis marsupialis</i>)
zalophi	Dailey MD	1980	Demodex zalophi sp.nov. (Acari: Demodicidae) from Zalophus californianus, the California sea lion	Acarologia (Paris)	California sea lion (Zalophus californianus)
bicaudatus	Kniest FM	1981	Parasites of Western Australia. XIII. A new species of demodicid mite from the meibomian glands of the bat Macroglossus minimus	Records of the Western Australian Museum	Long-tongued nectar bat (Macroglossus minimus)
leucogasteri	Hughes SE	1981	Demodex leucogasteri n.sp. from Onychomys leucogaster - with notes on its biology and host pathogenesis	Acarologia (Paris)	Northern grasshopper mouse (Onychomys leucogaster)
macroglossi	Desch CE	1981	A new species of demodicid mite (<i>Acari: Prostigmata</i>) from Western Australia parasitic on <i>Macroglossus minimus</i> (<i>Chiroptera: Pteropodidae</i>)	Records of the Western Australian Museum	Long-tongued nectar bat (Macroglossus minimus)
folliculorum sinensis	Xie H-X	1982	Taxonomy of the family <i>Demodicidae</i> and a new subspecies (Acarina: Demodicidae)	Acta Zootaxonomica Sinica	Human (Homo sapiens)

Species	Author	Year	Publication	Source	Species retrieved
huttereri	Mertens L	1983	Demodex huttereri spec.nov. (Acarina: Prostigmata: Demodicidae) from the meibomian glands of Apodemus agrarius (Rodentia: Muridae)	Bonner Zoologische Beiträge	Striped field mouse (Apodemus agrarius)
peromysci	Lombert H	1983	Demodex peromysci n.sp. (Acari: Prostigmata: Demodicidae), from the meibomian glands of Peromyscus leucopus (Rodentia: Cricetidae)	Journal of Medical Entomology	White-footed mouse (Peromyscus leucopus)
foveolator	Bukva V	1894	Demodex foveolator sp.n. (Acari: Demodicidae), a new epidermis-dwelling parasite for <i>Crocidura suaveolens</i> (Pallas, 1821)	Folia Parasitologica	Lesser white-toothed shrew (Crocidura suaveolens)
intermedius	Lukoschus FS	1984	Demodex intermedius sp.nov. (Acarina: Prostigmata: Demodicidae) from the meibomian glands of the tree-shrew Tupaia glis (Mammalia: Scandentia)	Malayan Nature Journal	Common tree-shrew (<i>Tupaia glis</i>)
sabani	Desch CE	1984	A new demodicid (<i>Acari: Demodicidae</i>) from the meibomian glands of southeast asian rats (<i>Rodentia: Muridae</i>)	Tropical Biomedicine	Striped field mouse (Apodemus agrarius)
buccalis	Bukva V	1985	Demodex rosus sp.n. and D.buccalis sp.n. (Acari: Demodicidae) parasitizing the upper digestive tract of rodents	Folia Parasitologica	Bank vole (Myodes glareolus)
flagellurus	Bukva V	1985	Demodex flagellurus sp.n. (Acari: Demodicidae) from the preputial and clitoral glands of the house mouse, Mus musculus L.	Folia Parasitologica	House mouse (Mus musculus)
rosus	Bukva V	1985	Demodex rosus sp.n. and D.buccalis sp.n. (Acari: Demodicidae) parasitizing the upper digestive tract of rodents	Folia Parasitologica	Yellow-necked field mouse (Apodemus flavicollis)
tigris	Shi, X-Q	1985	A new species of the genus <i>Demodex (Acariformes:</i> Demodicidae)	Acta Zootaxonomica Sinica	South China tiger (<i>Panthera tigris</i> <i>amoyensis</i>)
ailuropodae	Xu, Ye-Hua	1986	A new species of the genus <i>Demodex (Acariformes:</i> Demodicidae)	Acta Zootaxonomica Sinica	Giant panda (Ailuropoda melanoleuca)

Species	Author	Year	Publication	Source	Species retrieved
aries	Desch CE	1986	Demodex aries sp.nov., a sebaceous gland inhabitant of the sheep, Ovis aries, and a redescription of Demodex ovis Hirst, 1919	New Zealand Journal of Zoology	Sheep (Ovis aries)
neoopisthosomae	Desch CE	1986	Two new species of <i>Demodex</i> (<i>Acari: Demodicidae</i>) from the meibomian glands of the tropical Old World bat, <i>Eonycteris</i> spelaea (<i>Chiroptera</i>)	International Journal of Acarology	Cave nectar bat (Eonycteris spelaea)
spelaea	Desch CE	1986	Two new species of Demodex (Acari: Demodicidae) from the meibomian glands of the tropical Old World bat, Eonycteris spelaea (Chiroptera)	International Journal of Acarology	Cave nectar bat (Eonycteris spelaea)
tauri	Bukva V	1986	Demodex tauri sp.n. (Acari: Demodicidae), a new parasite of cattle	Folia Parasitologica	Cattle (Bos taurus)
kutzeri	Bukva V	1897	Demodex kutzeri sp.n. (Acari: Demodicidae), an identical parasite of two species of deer, Cervus elaphus and C.nippon pseudaxis	Folia Parasitologica	Red deer (Cervus elaphus), and Vietnamese sika deer (Cervus nippon pseudaxis)
bantengi	Firda KE	1987	Demodex bantengi n.sp. from Bos javanicus (d'Alton) with notes on gross pathology (Acari: Demodicidae)	International Journal of Acarology	Banteng (Bos javanicus)
acutipes	Bukva V	1988	Observations on the morphology of the hair follicle mites (Acari: Demodicidae) from Cervus elaphus L., 1758 including descriptions of Demodex acutipes sp.n.	Folia Parasitologica	Red deer (<i>Cervus elaphus</i>)
mystacina	Desch CE	1989	Two new species of <i>Demodex</i> (<i>Acari: Demodicidae</i>) from the New Zealand short-tailed bat, <i>Mystacina tuberculata</i> Gray, 1843 (<i>Chiroptera: Mystacinidae</i>)	New Zealand Journal of Zoology	New Zealand lesser short- tailed bat (Mystacina tuberculata)
novazelandica	Desch CE	1989	Two new species of <i>Demodex</i> (<i>Acari: Demodicidae</i>) from the New Zealand short-tailed bat, <i>Mystacina tuberculata</i> Gray, 1843 (<i>Chiroptera: Mystacinidae</i>)	New Zealand Journal of Zoology	New Zealand lesser short- tailed bat (Mystacina tuberculata)
uncii	Desch CE	1993	A new species of hair follicle mite (Acari: Demodecidae) from the snow leopard, <i>Panthera uncia</i> (Schreber, 1775) (<i>Felidae</i>)	International Journal of Acarology	Snow leopard (<i>Panthera uncia</i>)

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cornei	Mason KV	1993	A new species of <i>Demodex</i> mite with <i>D. canis</i> causing canine demodecosis; a case report	Annual Members Meeting of the American Academy of Veterinary Dermatology and the American College of Veterinary Dermatology, San Diego	Dog (Canis familiaris)
cricetuli	Hurley RJ	1994	Demodex cricetuli: new species of hair follicle mite (Acari: Demodecidae) from the Armenian hamster, Cricetulus migratorius (Rodentia: Cricetidae)	Journal of Medical Entomology	Gray dwarf hamster (<i>Cricetulus migratorius</i>)
desmodi	Desch CE	1994	A new species of <i>Demodex</i> Owen, 1843 (<i>Acari: Demodecidae</i>) from the meibomian glands of the vampire bat <i>Desmodus</i> rotundus (E. Geoffroy, 1810) (<i>Chiroptera: Phyllostomidae</i> : <i>Desmodontinae</i>) from Surinam	International Journal of Acarology	Common vampire bat (Desmodus rotundus)
agrarii	Bukva V	1994	Demodex agraril sp. n. (Acari: Demodecidae) from cerumen and the sebaceous glands in the ears of the striped field mouse, Apodemus agrarius (Rodentia)	Folia Parasitologica	Striped field mouse (Apodemus agrarius)
artibei	Vargas M	1995	Description of two new species of the genus <i>Demodex</i> Owen, 1843 (Acari: Demodecidae) associated with Mexican bats	International Journal of Acarology	Aztec fruit-eating bat (Artibeus aztecus), and Mexican big-eared bat (Corynorhinus mexicanus)
mexicanus	Vargas M	1995	Description of two new species of the genus Demodex Owen, 1843 (Acari: Demodecidae) associated with Mexican bats	International Journal of Acarology	Aztec fruit-eating bat (Artibeus aztecus), and Mexican big-eared bat (Corynorhinus mexicanus)
neomydis	Bukva V	1995	Demodex neomydis sp.n. (Acari: Demodecidae) from the hair follicles of the Mediterranean water shrew, Neomys anomalus (Insectivora: Soricidae)	Folia Parasitologica	Mediterranean water shrew (Neomys anomalus)
norvegicus	Bukva V	1995	Demodex species (Acari: Demodecidae) parasitizing the brown rat, Rattus norvegicus (Rodentia): redescription of Demodex ratti and description of D. norvegicus sp. n. and D. ratticola sp. n.	Folia Parasitologica	Brown rat (Rattus norvegicus)
ratiicola	Bukva V	1995	Demodex species (Acari: Demodecidae) parasitizing the brown rat, Rattus norvegicus (Rodentia): redescription of Demodex ratti and description of D. norvegicus sp. n. and D. ratticola sp. n.	Folia Parasitologica	Brown rat (<i>Rattus norvegicus</i>)
ursi	Desch CE	1995	A new species of <i>Demodex</i> (<i>Acari: Demodecidae</i>) from the black bear of North America, <i>Ursus americanus</i> Pallas, 1780 (<i>Ursidae</i>)	International Journal of Acarology	American black bear (<i>Ursus americanus</i>)

Species	Author	Year	Publication	Source	Species retrieved
bisonianus	Kadulski S	1996	Demodex bisonianus sp. nov. (Acari, Demodicidae) a new parasite of the bison (Bison bonasus L.)	Wiadomosci Parazytologiczne	European bison (<i>Bison bonasus</i>)
nycticeii	Desch CE	1996	Demodex nycticeii: a new species of hair follicle mite (Acari: Demodecidae) from the evening bat, Nycticeius humeralis (Chiroptera: Vespertilionidae)	International Journal of Acarology	Evening bat (<i>Nycticeius humeralis</i>)
sinocricetuli	Desch CE	1997	Demodex sinocricetuli: new species of hair follicle mite (Acari: Demodecidae) from the Chinese form of the striped hamster, Cricetulus barabensis (Rodentia: Muridae)	Journal of Medical Entomology	Chinese striped hamster (Cricetulus barabensis)
gatoi	Desch CE	1999	Demodex gatoi: new species of hair follicle mite (Acari: Demodecidae) from the domestic cat (Carnivora: Felidae)	Journal of Medical Entomology	Cat (Felis domesticus)
dasypodi	Desch CE	2002	First description of a hair follicle mite from the host order Xenarthra: Demodex dasypodi n. sp. (Acari: Demodecidae) from the nine-banded armadillo, Dasypus novemcinctus Linnaeus, 1758 (Dasypodidae)	International Journal of Acarology	Nine-banded armadillo (Dasypus novemcinctus)
phocidi	Desch CE	2003	Description of a hair follicle mite (<i>Acari: Demodecidae</i>) parasitic in the earless seal family <i>Phocidae</i> (<i>Mammalia: Carnivora</i>) from the harbor seal <i>Phoca vitulina</i> Linnaeus, 1758	International Journal of Acarology	Harbor seal (<i>Phoca vitulina</i>)
injai	Desch CE	2003	Demodex injai: a new species of hair follicle mite (Acari: Demodecidae) from the domestic dog (Canidae)	Journal of Medical Entomology	Dog (Canis familiaris)
macaci	Karjala Z	2005	First description of a new species of <i>Demodex (Acari</i> : Demodecidae) from rhesus monkey	Journal of Medical Entomology	Rhesus macaque (Macaca mulatta)
idopoud	Desch CE	2006	Two new species of <i>Demodex</i> Owen, 1843, the hair follicle mites (<i>Demodecida</i> e), from the dzungarian hamster, <i>Phodopus sungorus</i> (Pallas, 1773) (<i>Rodentia: Muridae</i>)	International Journal of Acarology	Djungarian hamster (<i>Phodopus sungorus</i>)
Sungori	Desch CE	2006	Two new species of <i>Demodex</i> Owen, 1843, the hair follicle mites (<i>Demodecidae</i>), from the dzungarian hamster, <i>Phodopus sungorus</i> (Pallas, 1773) (<i>Rodentia: Muridae</i>)	International Journal of Acarology	Djungarian hamster (<i>Phodopus sungorus</i>)

Species	Author	Year	Publication	Source	Species retrieved
tortellinioides	Desch CE	2006	Demodex tortellinioides n.sp. (Acari: Demodecidae) from the agile antechinus, Antechinus agilis Dickman, Parnaby, Crowther and King, 1998 (Marsupialia: Dasyuridae) in Australia	International Journal of Acarology	Agile antechinus (Antechinus agilis)
corniculatus	Izdebska JN	2012	A new <i>Demodecidae</i> species (<i>Acar</i>) from the yellow-necked mouse <i>Apodemus flavicollis</i> (<i>Rodentia: Muridae</i>) - description with data on parasitism	The Journal of Parasitology	Yellow-necked field mouse (Apodemus flavicollis)
microti	Izdebska JN	2013	Demodex microti n.sp. (Acari: Demodecidae) in Microtus arvalis (Pallas) (Rodentia, Cricetidae) with a checklist of the demodecid mites of cricetids	Systematic Parasitology	Common vole (<i>Microtus arvalis</i>)
gracilentus	Izdebska JN	2013	A new species of <i>Demodex</i> (<i>Acari: Demodecidae</i>) with data on topical specificity and topography of demodectic mites in the striped field mouse <i>Apodemus agrarius</i> (<i>Rodentia: Muridae</i>)	Journal of Medical Entomology	Striped field mouse (Apodemus agrarius)
auricularis	Izdebska JN	2014	Demodex auricularis sp.nov. (Acari: Demodecidae) from the ear canal of the European wood mouse Apodemus sylvaticus (Rodentia: Muridae)	International Journal of Acarology	Wood mouse (Apodemus sylvaticus)
ponderosus	Izdebska JN	2014	New species of <i>Demodex</i> (<i>Acari: Demodecidae</i>) with data on parasitism and occurrence of other demodecids of <i>Rattus</i> norvegicus (<i>Rodentia: Muridae</i>)	Annals of the Entomological Society of America	Brown rat (<i>Rattus norvegicus</i>)
lutrae	Izdebska JN	2014	Demodex Iutrae n. sp. (Acari) in European otter Lutra Iutra (Carnivore: Mustelidae) with data from other demodecid mites in carnivores	Journal of Parasitology	European otter (<i>Lutra lutra</i>)
conicus	Izdebska JN	2015	A new species of the genus <i>Demodex</i> Owen, 1843 (Acari: Demodecidae) from the ear canals of the house mouse <i>Mus</i> musculus L. (Rodentia: Muridae)	Systematic Parasitology	House mouse (Mus musculus)