Diffuse cutaneous candidiasis in a dog. Diagnosis by PCR-REA

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Summary

The authors describe a clinical case of cutaneous candidiasis in a dog with dermatological lesions, characterized by persistent alopecia, crusts, ulcers and scales. Predisposing factors such as the use of corticosteroids, the concomitan presence of an autoimmune disease (*pemphigus foliaceus)* and an infection of ehrlichiosis caused by *Ehrlichia canis* were observed.
Histopathological findings included signs of orthokeratotic hyperkeratosis, moderate follicular keratosis and light epidermic acanthosis. The reactive process included an infiltrative superficial dermatitis and a mural folliculitis with prevalent participation of macrophages and lymphocytes.
The application of PCR-Restriction Enzyme Analysis (REA) method on cutaneous specimens in veterinary medicine is an extremely interesting diagnostic tool. Its use, together with other techniques, such as mycologic, cytologic and histological examinations, allowed us to identify *Candida albicans* as aetiological agent in this particular case.

Key words Dog, *Candida albicans,* Cutaneous candidiasis, Molecular diagnosis, PCR-REA

Candidiasis cutánea difusa en un perro. Diagnóstico por PCR-REA

Resumen

Los autores describen un caso clínico de candidiasis cutánea en un perro que mostraba lesiones dermatológicas distribuidas sobre toda la superficie cutánea, caracterizada de una persistente alopecia, cotras, úlceras y escamas.
Los factores predisponentes incluían el uso de corticosteroides, la presencia concomitante de una patología autoimmune como el pénfigo foliáceo y de una patología infecciosa como la erlichiosis de la *Ehrlichia canis*. Los relieves histológicos evidenciaron signos de hiperqueratosis ortoqueratósica, moderada queratosis folicular y una ligera acantosis epidérmica. El proceso reactivo incluyó una dermatitis superficial infiltrativa y una foliculitis mural con una predominante participación de macrófagos y linfocitos.
La aplicación del método de reacción en cadena de la polimerasa – análisis con enzimas de restricción (PCR-REA) en las biopsias cutáneas en Medicina Veterinaria es un instrumento diagnóstico muy interessante. Su utilización, además de las técnicas usuales de micología, citología e histología, ha llevado a la identificación de *Cándida albicans* como agente etiológico.

Palabras clave Perro, *Candida albicans*, Candidiasis cutánea, Diagnóstico molecular, PCR-REA

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Superficial fungal skin infections in dogs include dermatophytosis, important for zoonotic implications, *Malassezia* dermatitis, more commonly recognized at the present time than in the past, and candidiasis [24]. Cutaneous candidiasis is an uncommon disease in dogs and *Candida albicans* is recognized as aetiological agent in primis [2,4,8,11,20]; in canine dermatitis *Candida parapsilosis* and *Candida guilliermondii* have also been involved [5,17].

C. albicans has a distinct predilection for mucosal surfaces and areas of mucocutaneous junctions of warm-blooded animals where it resides as commensal. Its biotope is the digestive system (where it is present as a minor member of the microbial flora), but under particular conditions, *C. albicans* becomes an opportunistic pathogenic micro-organism which may produce serious local infection and/or systemic invasion of the internal organs (such as kidney, liver, lungs, meninges or heart) [7,9,10,18].

Genetic (phagocytosis and T lymphocyte deficits), physiological (age, pregnancy), nutritional (vitamin deficiencies, severe malnutrition), pathological (endocrinopathies, immunodeficiency states) and iatrogenic (antibiotic, cortisonic, cytotoxic, immunosuppressor treatments) factors, operating individually or in combination, can predispose the passage from its commensal state to a pathogenic role. *C. albicans* has been associated with clinical cases in dogs with the involvement of different sites: external ear [1,14,19], the perineum, the nail folds, the oral mucosa, the cornea [13] the urinary tract [3].

C. albicans is not a member of the normal skin flora and its presence is always the expression of a direct responsibility of a pathologic state and of its intrinsic pathogenicity. The aim of this study was to describe an interesting clinical case of a diffuse canine dermatitis by *C. albicans* which was identified by means of the usual morpho-biological characterizations, and in bioptic specimens from the cutaneous lesions, by molecular biological techniques such as PCR-Restriction Enzyme Analysis (REA).

Materials and methods

*Case history***.** A three year-old male Pit Bull was regularly vaccinated and was living with his owners in a house-garden. At the age of eighteen months the dog showed signs of dermatitis on the face characterized by crusts, scales and partial itching. The bioptic examination led to the diagnosis of *pemphigus foliaceus* and the dog was subjected to cortisonic therapy for four months, which was then suspended due to the owner's decision. Two months later, the dog showed a serious aggravation of general conditions with anorexy, seediness, depression, anaemia and dehydration. At the same time, the dermatological lesions appeared on the whole cutaneous surface with alopecia, crusts, ulcers and scales. According to the clinical pattern, a treatment based on the use of griseofulvin was chosen. The dog was presented to the Veterinary Practice 45 days later and died after five days from the hospitalization.

Mycologic examination. Fungal cultures were made from lesions occurring in the back, abdomen, legs and from exudate taken from the bottom of the ulcer; physiologic solution used for the bioptic samples transfer was also incubated. The samples were cultured on Sabouraud's dextrose agar containing antibacterial antibiotics (20 I.U. penicillin and 40 µg/ml streptomycin) at 25 °C and on modified Dixon's agar at 32 °C for two weeks.

The identification of the yeast species was made considering the assimilation and fermentation properties with different biochemical sources [12]. Germ-test tube was also performed.

Cytologic examination. Different samples from ulcerative lesions of the skin were taken and smeared on a microscope slide for direct microscopic examination and then stained with May-Grunwald Giemsa (MGG).

Histological examination. The skin biopsies were fixed in 10% formalin, serially sectioned and stained with haematoxylin/eosin, PAS (Periodic Acid Schiff) reaction and GMS (Gomori Methenamine-Silver).

IFA Test for ehrlichiosis and leishmaniasis. Serum antibodies to *Ehrlichia canis* were determined by the indirect fluorescent antibody test (IFAT), developed by Ristic et al. [23], using infected monocytes by 50% as the source of antigen (Rhone-Mérieux, France). The antibodies to *Leishmania donovani* var. *infantum* were detected and quantified by the IFAT using promastygotes, cultured in Tobie's medium modified by Evans and fixed on multispot glasses, as antigen [21].

PCR-REA (Restriction Enzyme Analysis) procedure. DNA was extracted from a formalin fixed paraffin embedded biopsy specimen (10 mm), taken from the cutaneous lesion, as elsewhere described [25]. An aliquot (5 ml) of the aqueous DNA solution was submitted for PCR to amplify the yeast *ERG 11* gene (previously known as P450L1A1). The primers used in the PCR assay were $P45O_1$ (5'-ATGACTGATCAAGAAATYGCTAA-3') and $P450_2$ (5'-TAACCTGGAGAAACYAAAAC-3') that amplify a gene fragment of 300 to 350 bp, encoding a highly conserved region from the active site of the enzyme P450 lanosterol- 14α-demethylase P450L1A1 [15]. DNA solution was added to 50 ml of the PCR mixture, containing 5 ml of a 10X PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 8 ml of 200 mM (each) deoxynucleoside triphosphates (dATP, dCTP,dGTP,and dTTP), 25 ng (each) of primers $P450_1$ and $P450_2$ and 1.25 U of Taq DNA polymerase (Roche Molecular Biochemicals, USA), and the remaining volume of sterile distilled water. The PCR, performed in a thermal cycler, consisted of the first cycle: 3 min of denaturation at 94 °C, 1 min of annealing at 49 °C, and 90 s of extension at 72 °C, followed by 40 cycles of 45 s of denaturation at 94 °C, 1 min of annealing at 49 °C, and 90s of extension at 72°C. Sterile water as a negative control, and *C. albicans* DNA as a positive control were added in the run. The PCR product was analysed by agarose gel electrophoresis (2%) in TAE buffer (40 mM Tris acetate, 1mM EDTA, pH 8.4), stained with ethidium bromide (0.5 µg/ml), and visualized with UV light. Then, the PCR product was subjected to restriction enzyme analysis (REA) by using *Hinc*II, *N*siI and *Sau*3A (Roche Molecular Biochemicals) according to the manufacturer's instructions. After digestion, the DNA was subjected to electrophoresis in an agarose gel 4% (3% Seakem GTG and 1% Nusieve) in TAE buffer at 70 V for 2 h at room temperature, stained with ethidium bromide and photographed under UV light. The REA pattern obtained identified the amplified fungal DNA as *C. albicans* [15].

Results

Mycologic examination. In all the cultured samples on Sabouraud's dextrose agar various white or cream-coloured yeast-like colonies were isolated. The microscopic

examination of all the isolated colonies revealed yeast cells (3 to 6 µm in diameter) and blastoconidia (budding cells). The germ-test tube gave positive results. The fermentation and assimilation tests made possible to classify all the tested colonies as *C. albicans*. The growth of lipid-dependent yeasts on modified Dixon's agar gave negative results.

Cytologic examination. The cytologic examination of direct smears showed a subacute inflammatory process with numerous yeast organisms, some of which phagocyted by phagocytes mononuclear cells (Figure 1).

Histological findings. The histological examination of cutaneous biopsies showed signs of orthokeratotic hyperkeratosis, moderate follicular keratosis and light epidermic acanthosis. In bioptic sections the fungi looked like round basophilic cells or as pseudohyphae, infiltrating superficial and deep layers of epidermis and the internal portion of isthmus-infundibular structures of the hair follicle (Figure 2). PAS reaction and GMS stain showed the morphological features of the microrganism and its strong affinity for the different cutaneous keratinic structures. The reactive process included an infiltrative superficial dermatitis and a mural folliculitis with the prevalent participation of macrophages and lymphocytes.

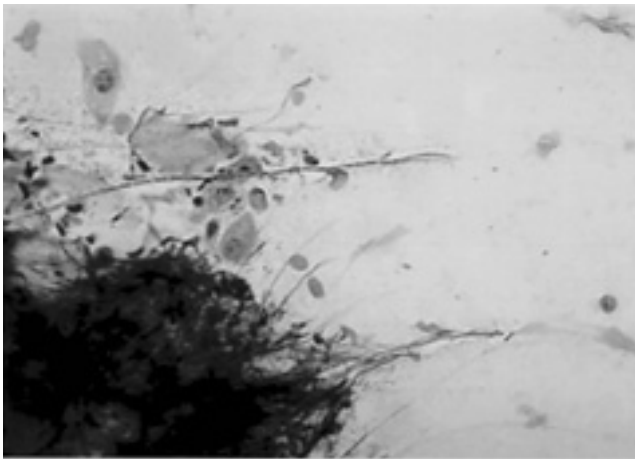

Figure 1. Cytologic examination: macrophages, mononuclear cells and epithelial cells are seen in association with pseudo-hyphal structures (May-Grunwald Giemsa stain, x40).

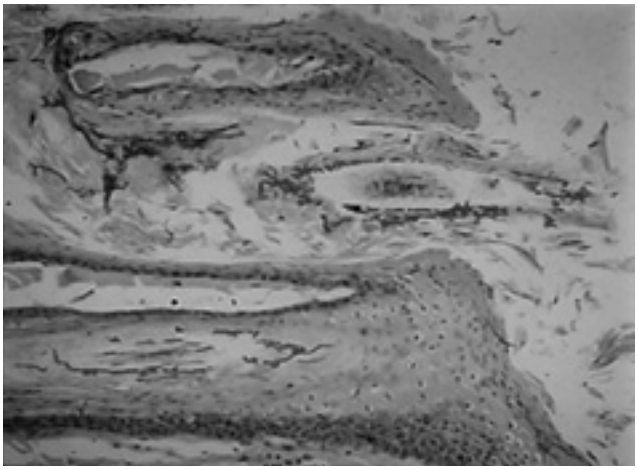

Figure 2. Histological finding of the skin: severe fungal colonization of the follicular infundibulum (PAS stain, x25).

PCR-REA. The restriction enzyme pattern of the amplified product, obtained from the cutaneous biopsy specimen, corresponded to that of *C. albicans*.

IFAT. The result of IFAT for leishmaniasis was negative whereas the reaction gave positive result for ehrlichiosis (1:800).

Discussion

The first candidiasis case discovered in dog dates back to 1949 [22]. The development of a clinical candidiasis form by *C.albicans* depends on the complex interplay between yeast virulence and host defence mechanisms.

Factors determining the intrinsic pathogenicity of *C.albicans* are the adherence and germination (crucial step to colonize abundantly and to invade the epithelial cells), toxin and enzyme production (which contribute to the process of invasion and damage of host cells) and resistance to inhibitory factors released by bacteria [6, 26].

The clinical case reported suggests interesting considerations: The concomitant presence of an autoimmune disease such as *pemphigus foliaceus* and of an infectious pathology as ehrlichiosis by *E. canis* (serologically diagnosed few days before the death) have determined a favourable condition to the invasion by *C. albicans*. *Pemphigus foliaceus* could have determined functional damage to the cutaneous immune system and in addition it may have induced cutaneous damage, whereas *E. canis* as infectious agent, has certainly "involved" the immune system, mostly cell-mediate immunity, representing the most specific antifungal barrier that the host-organism puts into action. The moderate immunologic response observed against *C. albicans* in the histopathologic findings, can be justified by the immunosuppressive effect of the corticosteroid treatment, an important predisposing factor.

The diffuse cutaneous lesions have a scaly-crusty pattern. The concomitant presence of a pustular ulcerative pattern may be initially associated to *pemphigus foliaceus* even if *C. albicans* may have contributed to ulcerating the epidermis. The final clinical finding is the result of the two aetiological entities. In natural infections *C. albicans* can also determine pseudomembraneous lesions (the archetypical lesion), inflamed, with irregular contour and lightly swollen, with infiltrations and vesicles, itching, preferentially located in the areas with cutaneous folds (interdigital spaces, groin, scrotum or in the perianal region). The persistent licking contributes to the extension of the lesions.

The histopathologic study showed a severe colonization of the follicular structures and of superficial and deep layers of epidermis through budding cells and intrusive pseudohyphae; colonization equal to that induced by dermatophytes. Two important considerations can be made: (i) *C. albicans* can be rightfully reported as the cause of folliculitis in comparison with the dermatophytes, *Demodex canis* or bacteria; (ii) to carry out a dermatological examination based only on the histopathologic and cytologic results, without mycologic cultures, is restrictive and difficult to prove the exact aetiological diagnosis for the morphologic similarity of some findings. The true prevalence of *C. albicans* in cutaneous infections can be underestimated and to perhaps ascribe to an incorrect diagnostic procedure; the dermatophytoses must be considered as a differential diagnosis in the case of these superficial fungal infections, even if they have a special tropism for keratinized tissues.

In order to develop a probable epidemiologic cycle, *C. albicans* is reported to be able to reach the skin from the endogenous source located in the alimentary tract (and therefore in the feces) with also the important presence of predisposing factors that can facilitate the process of invasion such as trauma and a local persistent moisture causing a disbalance of the superficial hydro-lipidic microfilm. Therefore, on one hand the intrinsic capacity of yeast to produce toxin and hydrolytic enzymes such as phospholipase, lysophospholipase and keratinolytic enzymes [6] and, on the other, a depressed cell-mediated immunity state can contribute to the process of invasion and damage of host-cell and to a serious diffuse cutaneous infection, respectively. The digestive locus of yeast *C. albicans* has been often observed from positive coproculture, in spite of apparent absence of digestive problems [8]. A non efficient cell-mediated immune response can give rise to the concomitant colonization of several species of fungi, as described by Wilkinson [27];

The molecular methods offer great applicative possibilities: taxonomic, epidemiologic and diagnostic definitions. In human medicine the identification of *Candida* DNA by a PCR method with subsequent restriction enzyme analysis (REA) has been recently developed and was carried out on blood samples belonging to patients with hematological malignancies [16]. The advantages are remarkable: the molecular method is significantly more sensitive than conventional tests; the response is quick; the method contributes to reveal subclinical or chronic infections with low levels of fungemia; the exact identification of the aetiological agent lead also to an application of a suitable and specific therapeutic protocol. In our case the administration per os, in the last period of life, of an unfit antimycotic (griseofulvin), not preceded by an appropriate diagnostic investigation, caused perhaps an aggravation of the clinical pattern.

The PCR-REA method is capable of identifying seven *Candida* species, including *Candida glabrata*, *Candida krusei* and *C. parapsilosis* which appear to be emerging as important new pathogens and *C. guilliermondii* known for its frequent resistance to amphotericin B. As already demonstrated [15], the PCR method was unable to amplify DNA extracted from fungi other than *Candida*, *Saccharomyces* and *Cryptococcus*, and human, bacterial or parasitic DNAs. In addition, the PCR-REA, due to its relative speed and sensitivity, is an useful tool for the management of *Candida* infections, especially when culture failed to reveal fungal growth from pathological specimens.

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