Disseminated *Penicillium radicum* infection in a dog, clinically resembling multicentric malignant lymphoma

**Gedissemineerde* Penicillium radicum* infectie bij een hond klinisch gelijkend op een multicentrisch maligne lymfoom**

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**ABSTRACT**

After detecting fungal organisms in smears of enlarged peripheral lymph nodes in a dog, determination was performed through culturing and genetic typing. The fungus was identified as *Penicillium radicum* by amplification of fungal DNA encoding of the ribosomal internal transcribed spacer region and comparison with DNA databases. In vitro susceptibility testing revealed multiresistance. In the literature, *P. radicum* is mentioned as a phosphate solubilizing agent used in agriculture for promoting plant growth. This is the first publication of a disseminated *P. radicum* infection in a dog. The generalized lymphadenopathy and hypercalcemia strongly resembled a multicentric lymphoma.

Hypercalcemia in granulomatous diseases, including disseminated fungal infections, is caused by activated macrophages possessing 1α-hydroxylase, which is able to convert 25-hydroxyvitamin-D into calcitriol. Although disseminated fungal infections are extremely rare in The Netherlands and Belgium, they should be included in the differential diagnosis of dogs with multicentric lymphadenopathy and hypercalcemia.

**SAMENVATTING**

In uitstrijkjes van vergrote perifere lymfeknopen van een hond werden schimmelstructuren aangetoond. De determinatie vond plaats via kweekmedia en een genetische typering. Het DNA dat codeert voor de “ribosomal internal transcribed spacer region” werd vermenigvuldigd, vergeleken met openbare DNA-databases en geïdentificeerd als overeenstemmend met dat van *Penicillium radicum*. In vitro gevoeligheidstesten toonden een multiresistente schimmel aan. Het gebruik van *P. radicum* in de landbouw wordt beschreven om plantengroei te stimuleren.

Door de gegeneraliseerde lymfeknooppelting en hypercalcemie vertoonde het ziektebeeld een sterke gelijkenis met een multicentrisch lymfoom. Hypercalcemie in granulomateuze ziektebeelden, inclusief gedissemineerde schimmelenfecties, wordt veroorzaakt door de omzetting van 25-hydroxyvitamine-D in calcitriol door geactiveerde macrofagen die 1α-hydroxylase bevatten. In Nederland en België komen systemische schimmelenfecties sporadisch voor maar ze dienen toch te worden opgenomen in de differentiaaldiagnose van honden met gegeneraliseerde lymfeknooppelting en hypercalcemie. Dit is de eerste publicatie van een gedissemineerde *P. radicum* infectie bij een hond.

**INTRODUCTION**

The diagnosis in animals of disseminated fungal infections, which are extremely rare in The Netherlands and Belgium, can be complicated. Most mycoses are easily confused with other disease entities, since the clinical picture of an animal with systemic mycosis is very unspecific. The causative agent needs to be promptly diagnosed and identified, usually by a combined microbiological and histopathological/cytological examination, if the infection is to be correctly treated and resolved. The principal route of infection for fungal diseases in dogs is inhalation, from which dissemination can occur. Occasionally, direct wound contamination and ingestion also play an important role in the pathogenesis of the disease, especially in histoplasmosis (Krohne, 2000).

The purpose of this report is to describe a case of...
disseminated canine fungal infection. To the best of our knowledge, this is the first report of a disseminated *P. radicum* infection in a dog. Furthermore, infection in humans with this species is not mentioned in an extensive review of invasive disease due to Penicillium species other than *P. marneffii* (Lyratzopoulos et al., 2002). In this series, *P. purpurogenum*, *P. brevicompactum* and *P. piceum* were among the most identified species. In humans, systemic infections with Penicillium species other than *P. marneffii* are rare, due to the inability of most non-marneffi species to grow at 37°C (Santos et al., 2006; Horré et al., 2001).

**CASE REPORT**

A 4-year-old male Dutch Braque with a body weight of 13.8 kg was presented in November 2007 with a mass, 3.5 cm in diameter, located in the left-ventral subcutaneous tissues of the neck. A complete physical examination showed no other abnormalities. A fine needle aspiration biopsy of the mass was performed and cytological examination revealed small lymphocytes, a moderate number of neutrophils, some plasma cells, and intracellular fungal hyphae in macrophages. A blood examination consisting of a complete blood count (CBC) and routine serum biochemistry profile showed no abnormalities, although no blood calcium concentration was determined at that time. The dog was treated with a total dose of 100 mg ketoconazole once daily per os for 3 weeks.

One week after end of therapy, the dog was submitted again with fever, anorexia and a stiff gait. The mass in the neck had doubled in size. Treatment was started with 5 mg/kg enrofloxacin once daily per os and 10 mg/kg amoxicillin twice daily per os. The mass was surgically removed, and post-operative incision showed the aspect of an abscess. Histopathological examination was not performed at that time. Wound healing was uneventful and antibiotic therapy was stopped 10 days after surgery.

Two months after surgery the dog developed signs of general fatigue, polydipsia and polyuria. On physical examination there was generalized lymphadenopathy and splenomegaly. Abnormalities noted on the serum biochemistry profile were a moderate hypercalcemia (3.32 mmol/l; reference range 1.98–3.00 mmol/l), slightly elevated creatinin (98 μmol/l; reference 70 μmol/l), and an increased ALAT (257 U/l; reference range 0–113 U/l). No abnormalities were observed on a CBC. A cranial mediastinal mass was surgically removed, and post-operative incision showed the aspect of these fungi in more detail, the smears of the lymph nodes and spleen that were temporarily stored were fixed overnight in 10% buffered formalin (pH 7.2) and stained with periodic acid Schiff reagent (PAS). The fungal hyphae that were ill-defined in the May-Grünwald Giemsa stain, their branching morphology was highly suggestive of phagocytized fungi (Figure 1). As a consequence, a presumptive diagnosis of a systemic mycosis was made on the basis of the cytological findings. In order to study the microscopic aspects of these fungi in more detail, the smears of the lymph nodes and spleen that were temporarily stored were fixed overnight in 10% buffered formalin (pH 7.2) and stained with periodic acid Schiff reagent (PAS). The fungal hyphae that were ill-defined in the May-Grünwald Giemsa stain demonstrated affinity for the PAS stain, and their presence in macrophages and multinucleated giant cells was clearly outlined (Figure 2).

Several fine needle aspirates were performed again aseptically from the prescapular lymph nodes and submitted for fungal culture. The aspirated material remained in the syringes and was inoculated in a laminar flow safety cabinet in Sabouraud agar and Malt extract agar within 30 minutes and incubated at 24°C and 37°C. After 5 days of incubation, fungal cultures were clearly visible (Figures 3 and 4). Microscopic characteristics of *Penicillium spp.* were seen after 14 days of incubation, which prompted a molecular approach, because classical morphological identification is inherently difficult. DNA was isolated from a 15-day-old pure colony using a standard protocol (Boom et al., 1990). The genomic DNA encoding the ribosomal internal transcribed spacer (ITS) region was amplified by using 5′-TCCGTAGGTGAACTTGCGG-3′ and 5′-TCTCCCTATTTATGTC-3′ primers. All amplicons were purified by using High Pure chemistry, and sequenced by using a MegaBACE DYEnamic ET Dye terminator kit, as suggested by the manufacturer. Reaction products were purified by ethanol pre-
The ITS sequence obtained for the isolate was compared to the public DNA databases by using the BLAST interface at http://www.ncbi.nlm.nih.gov/BLAST/ (Altschul et al., 1997), and proved to be 100% identical to previously reported *P. radicum* sequences. Molecular determination was confirmed by the CBS Fungal Biodiversity Center - KNAW, Utrecht, The Netherlands (http://www.cbs.knaw.nl), and the *P. radicum* isolate was deposited in the culture collection as CBS 122887.

The broth microdilution method was performed in duplo according to CLSI M38-A2 (Clinical and Laboratory Standards Institute, 2008). Briefly, a spore suspension of the isolate was made in 0.9% sterile saline with 0.05% Tween 40. The optical density was adjusted to 80 to 82% T with a spectrophotometer at 530 nm, and then the isolate was diluted 1000 times in RPMI medium (final concentration 1-5 x 10^3 CFU/ml).

One hundred microliters of the suspension were added to each well of a 96-well flat-bottom microtitration plate and incubated at 35°C for 48 hours. MIC was determined visually and spectrophotometrically as a ≥ 50% reduction of growth compared to that of the drug-free growth control for fluconazole. Also the lowest concentration of drug showing absence of growth was compared to that of the growth control for amphotericin Bi and the azoles. For caspofungin and anidulafungin, the MEC (minimum effective concentration) was determined microscopically as the lowest concentration in which abnormal, short and branched hyphal clusters were observed compared with the long, unbranched hyphal clusters that were seen in the growth control (Clinical and Laboratory Standards Institute, 2008). The MIC or MEC for amphotericin Bi, itraconazole, fluconazole, voriconazole, anidulafungin, posaconazole, isavuconazole, and caspofungin were 8, >16, >64, >16, 0.016, >16, 2, and >16 mg/L, respectively. The *P. radicum* isolate displayed resistance to the majority of the antifungals tested. Isavuconazole
had the lowest MIC (2 mg/L) compared with the other azoles and the MEC (0.016 mg/L) of anidulafungin suggested the isolate to be susceptible.

Meanwhile, the dog had difficulties in eating and was manually fed by the owner, but vomited almost after each meal. In expectation of the results of the antifungal susceptibility testing, therapy was started with 1 mg/kg maropitant® once daily as a subcutaneous injection and 5 mg/kg itraconazole® once daily per os. Vomiting stopped and three days after the beginning of therapy a moderate hypercalcemia (3.39 mmol/l; reference range 1.98–3.00 mmol/l) still remained.

Fourteen days after the beginning of itraconazole therapy, the condition of the dog still had not improved and the size of the lymph nodes had not decreased. The dog also developed uncontrolled muscular spasms in the head, an uncoordinated gait and an uncoordinated motoric activity when reaching out for food. Serum biochemistry profile abnormalities noted at that time included an increase in ureum concentration (14.8 mmol/l; reference range 1.98–3.00 mmol/l), a creatinin concentration of 4.17 mmol/l. The peripheral and medial iliac lymph nodes, as well as the spleen, were severely enlarged and the total condition of the dog rapidly declined. Euthanasia was performed on the owner’s request and no permission was obtained for necropsy.

DISCUSSION

Disseminated fungal infections in companion animals are extremely rare in The Netherlands and Belgium. In patients where such an infection clinically leads to generalized lymphadenopathy, splenomegaly and hypercalcemia, lymphoma should be included in the differential diagnostic work-up. This was especially true for the dog described in this case report, where there was no positive proof that the fungal organism detected on cytology specimens obtained from the mass in the neck was either the primary cause of or secondarily involved in the development of the abscess. Moreover, the time between the locally curative surgery and the development of the generalized lymphadenopathy justified putting lymphoma high on the list of differential diagnoses.

The genus Penicillium is among the most common fungi in the environment. Approximately 15 species are known to cause opportunistic human mycoses in immunocompromised patients (Lytratzopoulos et al., 2002). In dogs, several cases of disseminated Penicillium infection have been described in recent years. Among them are P. purpurogenum (Zanatta et al., 2006), P. commune (Kano et al., 2006) and P. brevicaespactum (Caro-Vadillo et al., 2007). A review of opportunistic fungal infections in 10 dogs noted one case of non-specified penicilliosis (Watt et al., 1995).

P. radicum is mentioned in the literature as a phosphate solubilizing fungus used in agriculture for plant growth promotion (Wakelin et al., 2007). The dog described in this article was living in an agrarian area, but no attempt was made to isolate the fungus from the dog’s environment, or to investigate the use of P. radicum in locally applied fertilizers.

A common theme in the clinical outcome of a fungal infection is the response of the immune system. If the inoculum is small and the animal is immunocompetent, the infection is usually limited to the port of entrance and resolves with few or no clinical signs. As in human patients, we can hypothesize that canine patients with systemic mycotic infections lack a proper immune response to the fungus. Although not convincingly proved, there are indications that immunosuppression – for example, through the neutropenia – in the dog described in this case report was the main cause of the fungus becoming invasive. Clinical signs in canine patients with disseminated fungal infections are primarily determined by the loss of function of the infected and inflamed organ systems, which causes non-specific clinical symptoms. However, draining skin tracts and lymphadenopathy have regularly been reported in several systemic fungal infections (Krohe, 2000).

Hypercalcemia has been well described in a variety of neoplastic and granulomatous diseases. Serum calcium concentration is tightly regulated by a complex system of calcitropic hormones. Parathyroid hormone (PTH) and the biologically active form of vitamin D, calcitriol or 1,25-dihydroxyvitamin D (1,25-(OH)2D3), are the most physiologically relevant calcitropic substances, with actions at the levels of bone, kidney and gut. Neoplasms such as lymphoma can alter the calcium homeostasis indirectly through the production of endocrine factors that result in hypercalcemia of malignancy. This paraneoplastic condition is most often due to the synthesis and release of parathyroid hormone-related peptide by neoplastic cells and the ensuing increase of osteoclastic bone resorption. Another mechanism for this hypercalcemia operates through the excess production of 1,25-dihydroxyvitamin D from extra-renal sources (Clines et al., 2005; Sharma, 2000).

Human patients with hypercalcemia during a dis-
25-hydroxyvitamin D (25-(OH)D3). 25-(OH)D3 is hydroxylated in the kidney to form 1,25-(OH)2D3. The renal 1a-hydroxylation of 25-(OH)D3 is the major recognized control point of vitamin D metabolism, responding to phosphate, PTH and calcitriol concentrations. Vitamin D receptors within renal proximal convoluted tubule cells are involved in an autocrine negative feedback loop whereby increased levels of 1,25-(OH)2D3 will down-regulate 1,25-(OH)2D3 production (Clines et al., 2005). Other known important extra-renal sites of calcitriol production are the placenta and granulomatous tissue (Clines et al., 2005).

However, hypercalcemia associated with granulomatous disease arises from an alteration of endogenous vitamin D metabolism. Macrophages activated in response to granulomatous inflammation are able to convert 25-hydroxyvitamin D3 produced by the liver into calcitriol in an unregulated manner by possessing the 1a-hydroxylase enzyme (Sharma, 2000; Dusso et al., 1991; Mellanby et al., 2006; Monkawa et al., 2007).

In companion animals, hypercalcemia related to granulomatous disease has been reported in disseminated histoplasmosis, blastomycosis, coccidiomycosis, tuberculosis and schistosomiasis (Mellanby et al., 2006; Dial, 2007; Dow et al., 1986). Animals with hypercalcemia related to granulomatous disease are expected to have high serum concentrations of calcitriol and of total and ionized calcium. However, their PTH values will be low. Serum calcium concentrations return to normal with successful treatment.

This case report illustrates that in veterinary medicine for the specific identification of generalized or unusual fungal disease, cytology, histopathology and culture of the suspected organism from fluids or tissue samples is to be supplemented by newer identification techniques such as PCR, to obtain a proper determination of the causative organism. In vitro susceptibility testing is of value to make targeted treatment possible. The isolate of this patient had the lowest MIC for antifungal activity for both fluconazole and itraconazole, two human antifungals not yet registered for this indication, and not a treatment option in this case.

### Commercial products mentioned in the paper

- **Amoxicilline**, Eurovet, Netherlands
- **Baytril**, Bayer BV, Netherlands
- **Cerenia**, Pfizer BV, Netherlands
- **Trisporal**, Janssen-Cilag BV, Belgium
- **Bristol-Myers Squib**, Woerden, The Netherlands
- **Columbia**, Intervet, Almere, The Netherlands
- **Baytril**, Bayer BV, Netherlands
- **Ampicillin**, Almene, The Netherlands
- **Eurogentec**, Seraing, Belgium
- **Roche Diagnostics**, Almere, The Netherlands
- **Amersham Biosciences**, Roosendaal, The Netherlands
- **Cerenia**, Pfizer BV, Netherlands
- **Trisporal**, Janssen-Cilag BV, Belgium
- **Bristol-Myers Squib**, Woerden, The Netherlands
- **Columbia**, Intervet, Almere, The Netherlands
- **Baytril**, Bayer BV, Netherlands
- **Ampicillin**, Almene, The Netherlands
- **Eurogentec**, Seraing, Belgium
- **Roche Diagnostics**, Almere, The Netherlands
- **Amersham Biosciences**, Roosendaal, The Netherlands
- **Cerenia**, Pfizer BV, Netherlands
- **Trisporal**, Janssen-Cilag BV, Belgium
- **Bristol-Myers Squib**, Woerden, The Netherlands

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**Uit het verleden**

**Op het eten van een kalfskop**

Maakt je klaar, maak je klaar,  
De kallefskop is gaar,  
Ei, hoor hem lekker koken!  
Mijn maagje danst op dat geluid,  
De kop die wil de ketel uit,  
Ik heb hem al geroken.

---

Wie zou niet, wie zou niet,  
Als hij ’t verhemelt ziet,  
Een stukje daarvan wensen?  
Wat zijn de harsens hagelwit,  
Zie of er ook een kei in zit,  
Als in de meeste mensen.

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Gans en kip, gans en kip,  
Kapoen en watersnip,  
Wie zou er naar je talen?  
Geen pluimgedierte, wild of tam,  
Geen osselong, geen hanekam,  
Mag bij de koppen halen

**Jan de Regt**

Dit zijn drie van acht strofen die de lof van kalfskop bezongen lang voordat er sprake was van BSE. De kop verscheen op zijn geheel op tafel: met tong, kaakspiertjes en hersenen! De kei in de hersenen is een alusie op het ‘keisnijden’ dat toen nog toegepast werd bij sommige geesteszieken. Het werkje werd neergepeld door Jan de Regt, een van die vrolijke poëten die Hollands’ Gouden Eeuw afsloten. Hij stierf in 1715. Geen verwarring mogelijk dus met zijn naamgenoot, de eerste anatomieprof aan de Gentse veeartsenijschool, geboren te Rotterdam in 1899.