Identification of *Prototheca* from the Cerebrospinal Fluid of a Cat with Neurological Signs

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Keywords:
Feline; Prototheca; Neurological symptoms; Infection; Alga; Diagnosis

1. Simple Summary

The genus *Prototheca* encompasses unicellular algae that are achlorophyllous and widespread in the environment. The genus is now included in the family Chlorellaceae, belonging to the order Chlorellales, which is included in the class, Trebouxiophyceae. *Prototheca* have repeatedly been reported to infect vertebrates. Cattle, dogs, and cats are the unique domestic animals in which *Prototheca* spp. have been reported to infect vertebrates. Cattle, dogs, and cats are the unique domestic animals in which *Prototheca* spp. have been reported to colonize different districts of the human body. Cats with protothecosis usually display a cutaneous disease, whereas dogs may develop both cutaneous and systemic forms. In this report, we identified molecularly *Prototheca* spp. in a cat with neurological signs. The animal presented a suspected diagnosis of multifocal lymphoma, and eventual immunological disorders/suppression likely triggered systemic diffusion of the achlorophyllic alga. Despite protothecosis not being regarded as a zoonosis, algal infections of animals should be recognized as indicators or sentinels of environmental risks for humans.

2. Abstract

*Prototheca* infections are rare in cats, and they are usually associated with cutaneous or subcutaneous infections by *P. wickerhamii*, with no evidence of neurological signs or systemic disease. In this study, we report the identification of *prototheca* in the cerebrospinal fluid (CSF) of a cat with neurological symptoms. Fourteen CSF samples were gathered from cats presented with neurological disease between 2012 and 2014. The inclusion criteria for the samples were an increase in CSF protein and cell number (pleocytosis), suggestive of an infectious inflammatory status of the central nervous system (CNS). Nine samples fulfilled the inclusion criteria (inflammatory samples), while five samples, used as control, did not (non-inflammatory samples). All the samples were screened molecularly for different pathogens associated with CNS disease in cats, including *prototheca*. Out of 14 CSF samples, only one inflammatory sample tested positive for *prototheca*. Upon sequence and phylogenetic analysis of the amplicon, the strain was characterized as *P. bovis*. This report is the first documented evidence of *prototheca* in the cerebrospinal fluid of a cat with neurological signs. *Prototheca* should be considered in the diagnostics.
procedures on the CNS of cats presented with infectious diseases.

3. Introduction

The Prototheca spp. consist of microscopical and unicellular organisms that are obliga-
tory heterotrophs because they lack chlo-
roplasts capable of photosynthesis [1-3]. Despite their yeast-like
morphology, based on genetic features, Prototheca spp. have been
classified as algae and included in the Prototheca genus closely
related to Chlorella genus in the family, Chlorellaceae [4]. Pro-
prototheca spp. are ubiquitous, may also colonize animal and human
gastrointestinal tracts, and have been occasionally reported in the
skin and nail beds of asymptomatic human patients [1,3,5]. Proto-
theca spp. Are also able to infect animals, but their specific path-
genic mechanisms of infection are yet to be elucidated. Several
Prototheca spp., i.e., P. cutis, P. miyajii, P. ciferrii, P. wickerhamii, P.
bovis, and P. blaschkeae, are able to infect both humans and
animals [6-8].

Protothecosis is a rare and occasional disease reported in humans
and domesticated as well as wild animals. Human and canine in-
fections have been described worldwide [9]. Mucosal contact,
ingestion, or traumatic introduction from contaminated fonts are
regarded as the most common sources of transmission of Proto-
theca spp. The algae penetrate the body via the respiratory or gas-
trointestinal tract and may then diffuse via ocular, cerebral, and
renal routes [10,11]. Over 95% of infections in human patients are
due to P. wickerhamii, with a small number of cases by P. bovis, P.
miyajii, P. blaschkeae, P. ciferri, or P. cutis [12-14]. In dogs, most
prototheca infections are caused by P. zopfii, with a few cases due
to P. wickerhamii [11,15].

Feline protothecosis is quite infrequent, either due to natural resist-
ance to infection or circumvention of environmental niches where
algae commonly establish. The exiguous recorded cases have all
been reported in clinically healthy adult cats with solid, non-
ulcer-
cuted, cutaneous or subcutaneous masses located on the forehead,
distal limbs, base of the tail, nose, or pinnae [16-19], and when the
isolates have been speciated, they have all been characterized as P.
wickerhamii [20]. Nasal localization of prototheca has also been
reported in cats [15,21]. The lack of regional lymphadenomega-
ly and clinical signs associated with systemic infection/disease
suggests that in cats, prototheca infection tends to be localized
[10], although a unique cat displayed new distant nodules several
months after excisional biopsy of an original solitary lesion [19].
Accordingly, unlike dogs, there is no evidence in the literature for
neurological signs or systemic symptoms associated with proto-
theca infection in cats [10].

4. Materials and Methods

4.1. Collection of Samples

Fourteen cerebrospinal fluid (CSF) samples were gathered from
cats with neurological disease at the veterinary clinics of Novara
and Arma di Taggia, Imperia, Italy, between 2012 and 2014. The
inclusion criteria for the samples were raised CSF protein and an
increase in the CSF cell number (pleocytosis), parameters suggest-
ive of an infectious inflammatory status of the central nervous
system (CNS). Pleocytosis in a CFS sample (lumbar punctate) was
categorized as positive with a protein fraction > 30 mg/dL or num-
ber of cells > 3 cells/µL, with a predominance of mononuclear
cells. Nine samples fulfilled the inclusion criteria (inflammatory
samples), while 5 samples did not and they were used as control.

4.2. Nucleic Acid Extraction

The nucleic acids were subjected to extraction from CSF sam-
ple employing the IndiSpin® Pathogen Kit (Indical Bioscience
GmbH, Leipzig, Germany), following the manufacturer’s instruc-
tions. Nucleic acid templates were stored at −70 °C until use.

4.3. Screening for Prototheca spp

Nucleic acid extracts were subjected to a PCR assay specific for the
18S rDNA of prototheca, using the forward primer Proto 18S-4F
(5’-GACATGGGAGGATTGACAGA- 3’) and the reverse prim-
er Proto 18S-4R-1 (5’-GACATGGCACCTGTATAC-3’) [22,23],
which amplify a PCR product of approximately 250 bp (Table 1).
Amplification was conducted using the Accuprime PCR Kit (In-
vitrogenTM Thermo Fisher Scientific, Shanghai, China) and P.
blaschkeae as a positive control. The bands were subjected to ex-
cision and purification by a QiaQuick Gel Extraction Kit (Qiagen
GmbH, Hilden, Germany), and the sequence was determined. Se-
quencing was performed at Eurofins Genomics (Vimodrone, Mi-
lan) laboratories. As an internal control, primers targeting the 28S
tRNA gene of the feline genome were used [33].

4.4. Quantitative Real Time PCR (qPCR), Specifically for P.
bovis

A qPCR specific for P. bovis was performed on samples testing
positive for Prototheca spp. Ten µL of sample DNA was combined
with the 15-µL reaction master mix (IQ Supermix; Bio-Rad Lab-
oratories SRL, Segrate, Italy), comprising 0.6 µmol/L of each prim-
er and 0.2 µmol/L of the probe (Table 1). Thermal cycling was
performed according to a previously described study [24].

4.5. Screening for Other Pathogens

Nucleic acid extracts were also screened for other feline patho-
gens, including feline infectious peritonitis, feline leukemia virus
(Felv), feline immunodeficiency virus (FIV), feline panleukope-
nia virus, rickettsia, neospora, toxoplasma, mycobacterium, and
bacterial 16S rDNA (Table 1).
<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Assay</th>
<th>Primers and Probes</th>
<th>Oligonucleotide Sequence</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Prototheca</em> spp.</td>
<td>PCR</td>
<td>Proto 18S-4F</td>
<td>5′-GACATGGCGAGGATTGACAGA-3′</td>
<td>[22]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5′-ATCACAGACCTGTATTAC-3′</td>
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<tr>
<td><em>Prototheca bovis</em></td>
<td>qPCR</td>
<td>PZg2F, SPZg2, PZg2R</td>
<td>5′-GACGATGATCCTAGTTATGTAC-3′</td>
<td>[24]</td>
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<td>5′-TATCAAGAAGCCTGAACGAC-3′</td>
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<tr>
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<td>qPCR</td>
<td>FCoV1128f, FCoV1200p, FCoV1229r</td>
<td>5′-GATTGATTTGCAAGTGCAATCTTT-3′</td>
<td>[25]</td>
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<td></td>
<td></td>
<td></td>
<td>5′-AACAATCATGACAGTGGATAC-3′</td>
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<td></td>
<td>5′-Fam-TCCATTGGTCTCGTCATAGCGGAG-Tamra3′</td>
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<td>PCR</td>
<td>118for, 119rev</td>
<td>5′-CTAACTCAAGTATGTCCCATG-3′</td>
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<td>5′-CTGGGGAGCCTGAGACGTCT-3′</td>
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<tr>
<td>feline immunodeficiency virus</td>
<td>PCR, nPCR</td>
<td>158for, 159rev, 160for, 161rev</td>
<td>5′-GAGTAGATACWTTGGTTCAAC-3′</td>
<td>[27]</td>
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<td>5′-CATCCTAATTCTTTGTTAAGC-3′</td>
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<td>5′-CAAATAGGATGGGTGAAY-3′</td>
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<td></td>
<td>5′-ACCATTCCWATAGCAGTRGC-3′</td>
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<tr>
<td>feline panleukopenia virus</td>
<td>qPCR</td>
<td>FPV/CPV-For, FPV-Pb, CPV-Pb, FPV/CPV-Rev</td>
<td>5′-ACACAAGATAAAAGCGACCTGTGGTAACTCAAA-3′</td>
<td>[28]</td>
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<td>5′-Vic-ATGGGAAATACAGACTATAT-MGB3′</td>
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<td>5′-Fam-ATGGGAAATACAGACTATAT-MGB3′</td>
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<td>5′-CAACCTCAGCTGTCTCATATAGT3′</td>
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<td>PCR</td>
<td>RSFG 877, RSFG1258</td>
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<td>5′-ATGGTCAAAAAGTACAGTGAACA-3′</td>
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<td>qPCR</td>
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<td>[31]</td>
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<td></td>
<td></td>
<td></td>
<td>5′-Fam-CCAGACGTTGATTTTCCGTGGTTCC-Tamra3′</td>
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<td></td>
<td>5′-TTCGTCCGTCGTAATATCGACG-3′</td>
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<tr>
<td>mycobacterium</td>
<td>PCR, nPCR</td>
<td>Myc For, Myc Rev, Myc NFor, Myc NRev</td>
<td>5′-CATGCAAGTCAAGCAGGGAAAG-3′</td>
<td>[32]</td>
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<td></td>
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<td>5′-CGGTGCTTCTTCTTCACCTA-3′</td>
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<td>5′-TACTCGAGTGGCGAAGGCTGGT-3′</td>
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<tr>
<td>bacterial 16S rDNA</td>
<td>PCR</td>
<td>B-V5, A-V6</td>
<td>5′-ATTAGATAACCCYGGTATGTTCC-3′</td>
<td>[33]</td>
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<td></td>
<td></td>
<td></td>
<td>5′-ACGAGCTGACGACARCCATG-3′</td>
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<tr>
<td>Internal control</td>
<td>PCR</td>
<td>feline 28S rDNA Fw, feline 28S rDNA Rv</td>
<td>5′-AGCAGAGAGG TGGTGAAAGAAG-3′</td>
<td>[34]</td>
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<td></td>
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<td></td>
<td>5′-AGGGAGAGCCCTAAATCAAGG-3′</td>
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</table>
4.6. Sequence and Phylogenetic Analyses

The online tool BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 15 September 2023) was used to find the highest nt identity in the NCBI database. Sequence editing was performed by the software package Geneious Prime v. 2021.2 (Biomatters Ltd., Auckland, New Zealand). Sequence alignments were performed by the MAFFT [34] plugin implemented in Geneious Prime. The best-fitting substitution model settings for the phylogeny were explored by the tool “Find the best protein DNA/Protein Models” of the MEGA X v. 10.0.5 software [35]. The evolutionary history was deduced by using the maximum-likelihood method, the Kimura 2-parameter model, a discrete gamma distribution to model evolutionary rate differences among sites (6 categories), and supplying statistical support with 1000 replicates. Bayesian inference and neighbor-joining phylogenetic analyses were also performed to explore the phylogeny of Prototheca spp.

5. Results

Fourteen CSF samples collected in this study were subjected to molecular screening for Prototheca spp. and a panel of feline pathogens. Out of 14 CSF samples, 1 sample (#628/14) tested positive for Prototheca spp. by PCR, and the sequence was determined. By BlastN analysis performed on a 250 bp sequence of 18SrDNA of Prototheca spp., strain #628/14 shared the highest nucleotide (nt) identity (100%) with P. zopfii var. hydrocarbonea strain UP-PT-P1 (EU439263). All the samples tested negative for feline infectious peritonitis, feline leukemia virus, feline immunodeficiency virus, feline panleukopenia virus, rickettsia, neospora, toxoplasma, mycobacterium, and bacterial 16S rDNA.

Partial 18SrDNA sequence (250 nt)-based phylogenetic analysis was performed using the sequence of Prototheca spp. generated in the study and the cognate sequences of the closest relatives retrieved from the NCBI database. Different phylogenetic approaches were explored for Prototheca spp., and similar topologies with slight differences in bootstrap values at the nodes of the tree were noticed. Accordingly, the maximum-likelihood (ML) tree was used. Upon ML analysis, strain ITA/2014/628 segregated with strains belonging to the P. bovis clade (Figure 1). Upon qPCR specific for P. bovis, sample #628/14 yielded 27 Ct.

The animal that tested positive for Prototheca spp. was a 9-year-old male domestic European cat, presented at the veterinary clinic with a 24 h history of seizures, incoordination, circling, and disorientation. Clinical pathological evaluation included a complete blood count and clinical chemistry panel. Blood analysis showed a marked increase in creatine kinase (9827 U/L, reference interval [ref.]: 91–326 U/L), alanine aminotransferase (517 U/L, ref.: 22–45 U/L), and aspartate aminotransferase (98 U/L, ref.: 21–41 U/L). The other parameters were not altered. Complete blood count parameters were within the reference interval. In addition, at the time of clinical examination, the animal tested negative for FIV and FeLV, using a quick test (SNAP FIV/FeLV Combo Test—IDEXX Laboratories). Abdominal ultrasound examination revealed multiple spleen and liver nodules. Fine needle biopsy specimens taken from the spleen and liver nodules revealed many lymphoid cells, and a suspect of lymphoma was included in the differential diagnosis.

Within 24 h, the clinical condition of the animal worsened, and the owner opted for the gentle suppression of the animal. Extreme care was employed to guarantee that death had happened prior to discarding the animal remains [36]. Before euthanasia, a CSF sample (#628/14) was collected with the permission of the owner, exclusively for research purposes.
Examination of the CSF displayed a distinct increase in total protein (2432 mg/L, range <300 mg/L) and cytological features consistent with marked mixed-type pleocytosis (2448 cells/µL, range <3 cells/µL), composed mainly of small and medium-sized lymphocytes with no red blood cells. The owner did not give permission for further investigations (i.e., necropsy) in the animal.

References


