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1 **Title page**

2 **Pythiosis: case report leading to new features in clinical and diagnostic management of**
3 **this fungal-like infection**

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34

35 **Abstract**

36 A 21-year-old man developed an infectious keratitis after swimming in Spain whilst wearing
37 contact lenses. Mycelial growth from a corneal sample suggested keratomycosis but a drastic
38 worsening of the patient's condition was observed on antifungal drugs. At day 38, the Pan-
39 fungal PCR identified *P. insidiosum*, which is an aquatic organism belonging to the oomycete
40 family. Based on recent literature, our patient was promptly prescribed systemic and local
41 antibiotic regimen and cure was ultimately achieved. In order to facilitate *P. insidiosum*
42 identification in future cases, we have generated the first MALDI-TOF MS reference
43 spectrum for *Pythium insidiosum*. This MALDI-TOF-MS reference spectrum is planned to be
44 deposited on an open-access-platform and should allow immediate identification of the
45 pathogen. Finally, this case report also demonstrates that *P. insidiosum* is emerging outside
46 tropical and subtropical areas. Clinicians and microbiologists should have better knowledge to
47 accurately manage and diagnose this sight-threatening infection.

48 **Keywords:** Pythiosis, *Pythium insidiosum*, keratitis, pan-fungal PCR, MALDI-TOF Mass
49 spectrometry

50

51 **Introducing pythiosis and *Pythium insidiosum*.** Pythiosis, also known as ‘swamp cancer’,
52 has only recently been identified in humans. Hosts become infected after contact between a
53 skin injury and zoospore-contaminated water. The form of pythiosis that develops (cutaneous,
54 vascular and ocular) depends on the exogenous inoculation site for this fungus-like pathogen.
55 A case of fatal disseminated pythiosis was recently described in an immunocompromised
56 patient with initial skin involvement (Hilton et al., 2016). Pythiosis is, thus, now considered
57 an emerging human infection (Gaastra et al., 2010; Hilton et al., 2016). The oomycete *P.*
58 *insidiosum* is the only etiologic agent of pythiosis in mammals. *P. insidiosum* is more closely
59 related to diatoms and algae, but, under the microscope, it appears as a fungus-like mycelium.
60 As pythiosis mimics fungal infections clinically and microbiologically, antifungal agents are
61 widely used for its treatment, resulting in a high rate of treatment failure (Agarwal et al.,
62 2017; Gaastra et al., 2010; Hilton et al., 2016; Krajaejun et al., 2004). Recent *in vitro* and *in*
63 *vivo* studies have suggested that some antibiotics may be more suitable than antifungal drugs
64 for the treatment of pythiosis (Jesus et al., 2015; Loreto et al., 2014).

65 We report a clinical case of ocular pythiosis due to the aptly named *P. insidiosum*,
66 which was initially misdiagnosed as keratomycosis. We propose new diagnostic and
67 therapeutic approaches to limit diagnostic errors and the prescription of ineffective treatments.

68 **Ocular pythiosis: the clinician’s view (figure 1- left side).** A 21-year-old patient
69 developed symptoms 13 days after water-based activities in the region of Cambrils in Spain
70 whilst wearing his monthly soft hydrophilic lenses. On his return, he presented with a corneal
71 abscess, with significant perilesional infiltrate. The patient was admitted to hospital and
72 initially treated with fortified antibacterial eye drops and anti-amebic treatments (fig. 1).
73 Local degradation was observed, necessitating emergency ultraviolet cross-linking, followed
74 48 hours later by transfixing keratoplasty (day 12-14). Microbiological analyses of the excised
75 cornea revealed the presence of a mycelium compatible with a filamentous fungus. The

76 treatment was switched to voriconazole, amphotericin B, hexomedine, PHMB and
77 dexamethasone/neomycin eye drops. After further local degradation, corneo-scleral
78 transplantation was performed (day 21). The dexamethasone drops, were stopped, and
79 treatment with 2% cyclosporine was introduced to prevent graft rejection. Rapidly, whitish
80 bodies were observed in the anterior chamber and treated by five surgical ablation associated
81 with injections of 0.1% voriconazole on days 29, 31 34, 36 and 38. Microbiological
82 examinations of the collections revealed a fungus-like mycelium that could not be identified
83 by conventional methods. A corneal fragment sent for pan-fungal PCR detected the presence
84 of *P. insidiosum* on day 38. Following this identification, and based on the most recent reports
85 concerning its antimicrobial drug susceptibility (He et al., 2016; Jesus et al., 2015; Loreto et
86 al., 2014), antifungals were stopped and antibiotics were prescribed: oral doxycycline and
87 clindamycin, and eye drops containing azythromycin and 2% cyclosporine. Since the
88 initiation of this dual antibiotherapy, no further local degradation has been observed.

89 **Ocular pythiosis: the microbiologist's view (figure 1- right side).** Initial
90 microbiological examinations were performed on material scraped from the cornea on days 0,
91 3 and 7: PCR for HSV and *Acanthamoeba*, and cultures for the detection of bacteria, fungi
92 and amebae were negative. The patient underwent a first keratoplasty on day 14. Two days
93 later, a small amount of mycelial growth was observed within the corneal fragment on the
94 agar plate, leading to a strong suspicion of keratomycosis. Surprisingly, none of the various
95 subcultures on Sabouraud-chloramphenicol agar plates yielded positive results, whereas
96 subculture in brain-heart-infusion resulted in the rapid growth of a large mycelium at 35°C.
97 All attempts to identify this mycelium on the basis of microscopic morphology or by MALDI-
98 TOF MS failed. Indeed, despite the high quality of the protein spectrum, it could not be
99 attributed to any of the microorganisms present in the Bruker Taxonomy (7815 entries),
100 Bruker Filamentous Fungi (364 MSP), NIH mold (365 profiles) or MSI fungal (> 22,000

101 spectra) databases. The corneal biopsy specimen and the mycelium were, therefore, sent for
102 pan-fungal PCR diagnosis on day 29. Biomolecular analyses were performed with panfungal
103 primers (ITS1/ITS4 and NL1/NL4) and sequencing results obtained on day 37 unambiguously
104 identified the species present as *P. insidiosum*.

105 **Discussion**

106 *Pythium insidiosum* has rarely been identified as the causal agent of infectious
107 keratitis, particularly in patients with no history of travel to tropical areas. There is, therefore,
108 no clear algorithm for the management of this mold-like infection. Cryotherapy, absolute
109 alcohol during surgery and antifungal agents administered topically or by injection have all
110 been used, but with poor success rates, and enucleation is frequent (up to 42%) (Agarwal et
111 al., 2017; Lelievre et al., 2015). Adjuvant immunotherapy with *P. insidiosum* antigen
112 injection (PIA) has also been described (He et al., 2016). Recent studies *in vitro* (Loreto et al.,
113 2014) and *in vivo* (Jesus et al., 2015) have highlighted the possible activity of antibiotics
114 against *P. insidiosum*. In particular, macrolides (azithromycin) and tetracyclines
115 (minocycline) have been shown to have synergistic activity *in vitro*, whereas antifungal drugs
116 are ineffective. Oral azithromycin was also found to be the most active treatment *in vivo*.
117 These results strongly suggest that systemic macrolide (+/- tetracycline) treatment should be
118 preferred over antifungal drugs as the first-line treatment for pythiosis. Ramappa *et al.*
119 successfully treated a presumptive case of *Pythium* keratitis with systemic and local regimens
120 of azithromycin and linezolid (Ramappa et al., 2017). Based on these findings, our patient
121 was prescribed this dual antibiotic regimen. However, this treatment was not initiated until 38
122 days. Similar long delays to the diagnosis of *pythiosis* were reported in all previous cases. The
123 delay was particularly long in this case because, despite the presence of sterile mycelium,
124 successive subcultures were negative. *Pythium* generally grows well on Sabouraud medium,
125 but it did not do so on a Sabouraud supplemented with chloramphenicol. Additional time was

126 also lost to the various attempts to identify this fungus-like organism by MALDI-TOF-MS,
127 because there is currently no reference spectrum for *P. insidiosum* in the various MSP
128 databases commonly used for microbial identification (Drissner and Freimoser, 2017).
129 Therefore, after confirmation by sequencing, we created a reference MSP on the Microflex
130 mass-spectrometer, according to Bruker's MSP creation protocol (v1.1). This reference
131 spectrum was added to our local database and can be shared on request. It is also planned to
132 be rapidly added to the MSI database ([https://biological-mass-spectrometry-
133 identification.com/msi/welcome](https://biological-mass-spectrometry-identification.com/msi/welcome)), serving as a free identification platform for MALDI-TOF
134 identification, to facilitate the diagnosis of *Pythium* infections in the future both at our
135 institute and at other healthcare (or veterinary) centers.

136 **Conclusion**

137 We can learn several lessons from this atypical case of keratitis. First, contrary to previous
138 suggestions, *P. insidiosum* does not seem to be limited to tropical and subtropical areas. Even
139 in the absence of travel to the tropics, this etiologic agent should be considered in cases of
140 suspected keratomycosis with water-related exposure, particularly if the infection is resistant
141 to antifungal treatments. Second, our findings suggest that antibiotic-supplemented media
142 should be avoided, whereas Sabouraud agar without antibiotics or yeast extract–peptone–
143 dextrose (YPD) should be used preferentially to ensure accurate diagnostic. Third, although
144 we have little hindsight for the moment, antibiotic treatment seems to be more effective
145 against *Pythium* than antifungal treatments and should be considered as a first-line therapy as
146 soon as the identification is confirmed. Finally, in this case, it was possible to achieve
147 definitive identification of the pathogen by sequencing. However, there is a need to develop
148 rapid unambiguous diagnostic tools to facilitate the identification procedure. For this purpose,
149 we have generated the first MALDI-TOF reference spectrum for *Pythium insidiosum*, which
150 we have made freely available.

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156 **Conflict of interest**

157 No conflict of interest

158 **Ethical Approval**

159 Not required.

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196

197

Figure legend

Figure 1: Pythiosis: chronology of events, the clinician's (left) and microbiologist's (right)

viewpoints. D: day; MSP: mass spectrum profile; HSV: herpes simplex virus; PHMB: polyhexamethylene biguanide. Phaco: phacoemulsification; DMEK: Descemet membrane endothelial keratoplasty. TK : transfixiant keratoplast

MEDICAL AND SURGICAL CARES

MICROBIOLOGY

D-13: Probable contamination



Hexamidine & PHMB
Vancomycin & amikacin



Cross linking &
Transfixing keratoplasty

Hexamidine & PHMB
Dexamethason / Neomycin



Spectacular TK
degradation



Corneo-scleral graft

Voriconazole IV
Ciclosporin 2%
Amphotericin B
Voriconazole



Anterior chamber
washout
& voriconazole injections



Oral doxycyclin
Oral clindamycin
Azythromycin
Ciclosporin 2%



No sign of relaps of the
infection
Deficiency of the corneo-
scleral graft
Phaco DMEK ?

D...

D_{1,3,7} Corneal scraping

Sterile cultures

- Bacterial & mycological

Negative PCR

- Acanthamoeba & HSV

D₁₄ Corneal fragments

Mycelium growth

- Corneal biopsy
- BHL media

Sterile cultures
Negative PCR

- Bacterial
- Acanthamoeba

D₂₉ Pan-fungal PCR

GenBank - *Pythium insidiosum*

Pythium insidiosum isolate CBS 174.05.200 large subunit ribosomal RNA gene, 5' sequence (AF020422.1) length: 1014, Number of Matches: 1

Accession	Score	E-value	Max ident	Start	End	Ident
GenBank: AF020422.1	99.8	0.0	100%	1	1014	1014
GenBank: AF020422.1	99.8	0.0	100%	1	1014	1014
GenBank: AF020422.1	99.8	0.0	100%	1	1014	1014
GenBank: AF020422.1	99.8	0.0	100%	1	1014	1014

D38: *P. insidiosum* identification

D₅₀ *P. insidiosum* MSP creation
MALDI-TOF identification

