Curr Fungal Infect Rep (2013) 7:198–208
DOI 10.1007/s12281-013-0144-z

CLINICAL LAB ISSUES (M BRANDT, SECTION EDITOR)

The Mammalian Pathogenic Oomycetes

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Published online: 5 June 2013

Abstract The oomycetes are fungal-like microbes similar to those found within some members of the kingdom Fungi. Although these two groups of microbes share morphological features, there are several contrasting differences: a) phylogenetic analysis placed the oomycetes basal to plants and green algae; b) oomycetes lack ergosterol in their cytoplasmic membrane; c) chitin is not the main compound in the cell wall of oomycetes; and d) asexual reproduction in the oomycetes occurs by the development of sporangia containing numerous biflagellate zoospores. *Pythium insidiosum* was considered to be the only oomycete pathogenic for mammals. However, in 1999, Grooters reported that several dogs were diagnosed with an unusual oomycete in the genus *Lagenidium* causing extensive cutaneous and subcutaneous infections. Thereafter, the infection has been also reported in humans and cats, and it could possibly affect other mammalian species as well. This review highlights the epidemiological, clinical and pathological features, as well as the diagnosis and management of the infections caused by this unique group of mammalian pathogenic oomycetes.

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Keywords Pythiosis · Lagenidiosis · *Pythium insidiosum* · *Lagenidium* species · *L. caninum* · *L. karlingii* · Oomycetes · Oomycosis · Fungal-like · Aquatic fungi · Parafungal microbes

Introduction

The class oomycetes (peronosporomycetes) is a group of fungal-like (parafungal) microbes located basal to the plants and closely related to the green algae in the kingdom Straminipila (Stramenopila) [1•]. Members of this group have been known for quite some time as "the aquatic fungi" because they possess tubular bodies (hyphae) with few septa, similar to those encountered in the Zygomycetes, kingdom Fungi [2], and they are usually located in aquatic environments [2, 3•, 4]. However, the main feature of the oomycetes is the development of sporangia with the formation of biflagellate zoospores in wet ecological niches [3•, 4]. The true taxonomic and phylogenetic position of the oomycetes in the tree of life has been only recently studied in detail [5, 6]. With the use of molecular methodologies, this group of saprotrophic and pathogenic organisms was found to be phylogenetically distant to the fungi, but to be part of the members in the kingdom Protist (Chromista) [1•]. This taxonomic definition of the oomycetes is rather simplistic. Doubtlessly, the phylogenetic placement of these microbes in the tree of life is more complex than it seems at first glance.

Although some oomycetes are saprotrophic in nature, the capacity of some species to infect a wide variety of hosts is a characteristic of the class. This group of microbes has been reported to cause infection in plants (*Pythium*, *Phytophthora*, and others) [7, 8]; animals, such as: insects (*Lagenidium*, *Pythium*) [2, 9••], arthropods (*Lagenidium*, *Myxozitium*, and others) [2], fish (*Achlya, Saprolegnia, Pythium*) [10–12], crab, lobster and shrimp (*Haliphthorus*, *Halodaphnea*) [2], algae (*Lagenidium*) [2], rotifers (microscopic animals) (*Lagenidium*)

[2], birds (*P. insidiosum*) [13], mammals (*Lagenidium* and *Pythium*) [3•, 14••, 15, 16]; and algae (*Lagenidium*) [2]. Mendoza [9••] recently speculated that the ability of some oomycetes to cause disease in various vertebrate animals may reside in the fact that they have been practicing with invertebrate animals for millions of years. Thus, through their evolutionary history of previous contacts with vertebrate animals, they probably acquired putative virulence factors to affect such animals. Although oomycetes likely developed several virulence factors to affect mammals, the destructive nature of the disease itself indicates that they are not good parasites and that they are still evolving. Since oomycetes are not true fungi, they lack ergosterol in their cytoplasmic membrane, and thus these species are intrinsically resistant to most antifungal drugs targeting this pathway. This is a challenge for clinicians, since pythiosis and lagenidiosis are life-threatening infections in all species [17••]. In this review, we will cover infections caused by several oomycetous species in the genera *Pythium* (*Pythium insidiosum*) and *Lagenidium* spp that affect mammalian hosts.

Pythium Insidiosum

The infections caused by *Pythium insidiosum* are known under the term pythiosis, which was first introduced by Chandler et al. in 1980 [18]. The disease has been also been referred to by names such as: bursatti, Florida horse leeches, granular dermatitis, summer sores, and swamp cancer [3•, 17••]. Some of these colloquial names are directly linked with epidemiological features of the disease and the fact that the oomycetes are located in aquatic environments. For example, bursatti means rain in India; and Florida horse leeches and swamp cancer are both related to the development of cutaneous ulcers in horses after they had entered wet areas in Florida, USA [17••, 18]. Pythiosis has been reported in numerous species of apparently healthy mammals, including cats, dogs, horses, sheep, humans [19–26], a California bird [13], and zoo-captive animals including: camels [27], big cats [28], and bears [17••]. So far, *P. insidiosum* has been recorded as the only species causing pythiosis. However, *P. aphanidermatum*, a typical plant pathogen, was isolated from a human case [29]. In addition, a strain studied in Africa from a dog with cutaneous pythiosis displayed a phylogenetic pattern different from that published by others [30•]. These findings could well indicate that more than one species could be involved in cases of pythiosis in mammals.

Geographical Distribution

The disease has been reported in tropical and subtropical areas, with the majority of cases occurring in the Americas, Asia, and Australia [17••, 31, 32]. In these areas, the species affected by *P. insidiosum* with greatest frequency are dogs and horses [17••, 24, 33]. The disease is sporadic in humans and other mammalian species [17••]. In the Americas, pythiosis has been reported in southern USA, with some cases in the northern States, and in practically all Latin American countries except Chile. In Africa, only one case in a dog has been recorded so far [30•]. Australia reported pythiosis early in the last century, mainly in horses [17••]. More recently, cases in dogs and humans have been also diagnosed in this region [17••]. Some of the first reports of pythiosis were recorded in the late 19th century by the Dutch in Indonesia, and by English veterinarians in India [9••]. The disease has also been diagnosed in Japan and in the Pacific Islands, including Indonesia, New Zealand, and Papua New Guinea.

Epidemiology and Experimental Infection

All oomycetes develop sporangia and zoospores to colonize new environments [1•, 2, 4, 9••]. Most oomycetes have a special tropism to their host tissues, used to complete their life cycle in nature. For instance, zoospores of typical plant pathogens such as *Pythium* spp and *Pythophthora* spp. have a special tropism for plant tissues. After they have reached their host, the zoospores attach to the plant, develop a germ tube, and actively penetrate the host [4, 7]. Experimental data have shown that *P. insidiosum* possesses a strong tropism for plant tissue as well as for mammalian open skin [4, 9••]. Thus, it has been postulated that this oomycete uses this strategy to locate mammalian hosts with skin injuries, when the host enters aquatic environments contaminated with *P. insidiosum*. Once the zoospores come in contact with a mammalian host, they lose their flagella, encyst, and secrete an adhesive-like material [4]. This secreted material keeps the encysted zoospore attached to the host, which increases its chance of survival. Stimulated by body temperature, the encysted zoospore develops a germ tube similar to the one produced during its life cycle in nature. The germ tube pointing toward the wound penetrates host tissues, causing pythiosis [4, 9••]. It is believed that most mammalian hosts are resistant to *P. insidiosum* infection, and only a few develop cutaneous, subcutaneous or systemic infections. The occurrence of the disease is unknown, mainly because it is not a reportable disease. According to data of the last 10 years, the disease seems to be more common in dogs and horses and very sporadic in humans and other animals [9••, 17••]. Thailand has reported more cases of human pythiosis than any other endemic area of the world. Interestingly, few cases in other animals have been recorded in this country [25, 26, 34•]. The disease has not been linked to immunocompromised hosts, but cases in thalassemic patients in Thailand have been mentioned [34•].

Infection can be experimentally reproduced only in rabbits [17••]. Several studies have been carried out to

reproduce experimental pythiosis in cats, cattle, dogs, horses, mouse, rats, and even humans, but without success. Amemiya [35], using strains of *P. insidiosum* recovered from horses in Japan with the disease, reported what early investigators already recorded in their experiments: the etiologic agent of granulomas in horses can only be reproduced in rabbits [17••]. These investigators found that rabbits were a good model for experimental investigation of pythiosis. We now know that the experimental model using rabbits can mimic the cutaneous and arterial disease observed in humans and other animals with the infection, and thus it is a good model to study this disease.

Pathogenesis, Clinical Features and Pathology

Once the zoospores of *P. insidosum* develop a germ tube and penetrate the mammalian host's tissues, the host will react with a strong inflammatory response [36•]. Pythiosis in mammals is usually diagnosed in apparently healthy hosts. We believe that the initial response to the invading hyphae could control the pathogen and actually could eliminate *P. insidiosum* from the tissue. This belief is supported by the occurrence of few pythiosis cases in humans or other animals in heavy endemic areas. As Mendoza and Newton [36•] mentioned, it is quite possible that susceptible humans and animals may harbor undetectable defects in genes related to key cell receptors that render these hosts susceptible to this mammalian pathogenic oomycete. If *P. insidiosum* cannot be eliminated by this initial response, the host's immune system triggers a typical Th2 response in the infected areas [17••, 36•]. The presence of numerous mast cells, eosinophil, neutrophils, lymphocytes, activated macrophages, and sometime giant cells, is typical of acute and chronic stages of the infection. The formation of huge pruritic ulcerate tissue is related to the number of eosinophil and mast cell degranulations over the invading *P. insidiosum* hyphae [36•]. This reaction leads to necrosis and further complicates the clinical picture of the host. The degranulation of eosinophils leads to the formation of eosinophilic material around the hyphae, known as the Splendore/Hoeppli phenomenon [14••, 20, 30•, 37, 38]. This phenomenon is extremely intense in horses, where small hard masses termed "kunkers" are formed [17••]. These masses contain eosinophil picnotic nuclei, cellular detritus, and healthy hyphae of the pathogen. Elevated titers of Th2 interleukins (IL4, IL10, and IL5) have also been detected in patients with pythiosis [39•].

The clinical features of pythiosis depend on the anatomically affected area. In humans, *P. insidiosum* can be found on superficial infections (keratitis) [40–42], subcutaneous and cutaneous pythiosis [31], and arterial pythiosis causing disseminated infections [23, 26, 34•, 39•], whereas in animals, subcutaneous and intestinal diseases are more common (Fig. 1) [14••, 24]. Superficial keratitis in humans has been reported in endemic areas, including a recent outbreak in Thailand [22]. In early stages, the infection is characterized by eye irritation and the formation of a small ulcer. Trauma and exposure to water events prior to the onset of symptoms are common, especially in contact lens users [41, 42]. As the infection progresses, slight fever, pain, photophobia, and layered hypopyon can be found [22, 40–42]. Subcutaneous pythiosis with extensive ulcerate tissue or large swelling with serosanguinous discharge can be found. The invasion of large arteries of the limbs is commonly diagnosed in Thai patients [23, 26, 34•, 39•]. This condition leads to obstruction of blood vessels, aneurysms, and gangrene of the affected limbs. Arterial pythiosis is a life-threatening condition, especially when *P. insidiosum* reaches the aorta [23, 32].

Pythiosis in animals is also a life-threatening infection. In dogs, *P. insidiosum* affects the subcutaneous tissues as well as the intestinal tract [17••, 24, 38]. Ulcerate tissue, swellings, and sinus tracts with the involvement of regional lymph nodes can be found in dogs. Intestinal dog pythiosis is characterized by large tumoral-like masses in the affected intestinal areas. Diarrhea, fever, lethargy, anorexia, weight loss and death are common in intestinal dog pythiosis [24]. In cats, pythiosis is a protracted disease, usually confined to subcutaneous tissue [19]. In horses, *P. insidiosum* has been observed to affect the subcutaneous tissues, and rarely other anatomical areas including bones [17••]. Itching of the infected areas and the formation of stony-like masses known as kunkers (see above) are typical of pythiosis in this species. No other mammalian species develops these structures in the infected areas.

The pathological changes in animals and humans include formation of extensive necrotic areas with the development of ulcerate tissue and/or tumoral-like masses with fistulas. With H&E staining, *P. insidiosum* hyphae are observed as transversal or longitudinal empty structures (called "hyphal ghost elements" by some) that do not stain well [15, 17••]. An eosinophilic precipitate is commonly found surrounding the invading hyphae (Splendore/Hoeppli phenomenon). This is in contrast to the Mucorales fungi, which seem to stain well with H&E and lack the Splendore/Hoeppli phenomenon around their hyphae. When stained with silver, *P. insidiosum* appears as slender, sparsely septate hyphae that are 5 to 10 μm in diameter [15].

Laboratory Diagnosis

The diagnosis of pythiosis includes the use of wet mounts, stains, serological assays, DNA testing, and culture. The collection of biopsied tissues from infected areas is common practice [15]. However, in cases of superficial keratitis, deep scraping of the ulcerated cornea is common [40, 41]. In horses with pythiosis, the collection of the stony masses known as kunkers is preferred [17••].

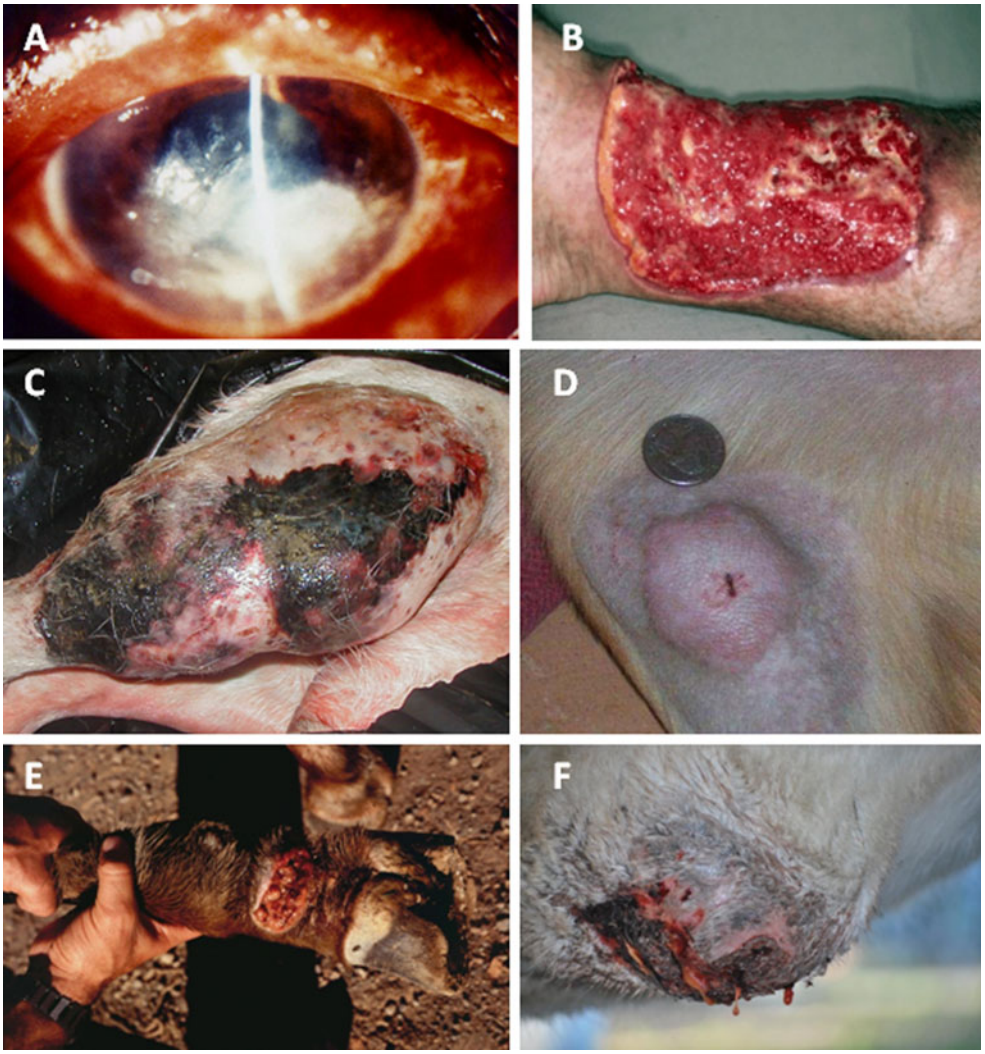

Fig. 1 Panels **a**–**d** show clinical features of infections caused by *Pythium insidiosum* in humans (**a** and **b**), dogs (**c** and **d**), and horses (**e** and **f**). In humans, the infections caused by this pathogenic mammalian oomycete include superficial infections of the cornea (keratitis) (Panel **a**, courtesy of Dr. Champrasiet) as well as subcutaneous ulcerated tissue (Panel **b**, courtesy of Dr. Bagagli). In dogs, the infection involves subcutaneous tissues (Panels **c** and **d**) as well as the intestinal tract. In horses, *P. insidiosum* tends to be limited to cutaneous and subcutaneous tissues (Panels **e** and **f**)

Wet Mounts and Stains In wet mount preparations, *P. insidiosum* appears as long hyaline tubular bodies 4 to 10 μm in diameter with few septa, very similar to the hyphal elements encountered in cases of mucormycosis and lagenidiosis (see below). The presence of Gram-positive short and long hyphal elements has been also reported in some cases of pythiosis [17••]. The organism has been also visualized in cytological preparations stained with Giemsa.

Serological Assays Several serological assays have been used to detect the presence of anti-*P. insidiosum* antibodies [14••, 43–45]. These include immunodiffusion (ID), Enzyme linked Immuno-Sorbent Assay (ELISA), Western blot (WB), and haemogglutination tests. ID is very specific but has a low sensitivity, whereas ELISA and WB are both very sensitive and specific. There are few places in the USA that test for ELISA anti-*P. insidiosum*. One such place is Pan American Veterinary Labs (http://pythium.pavlab.com/). This company offers ELISA and culture testing for pythiosis in all species. In addition, the detection in histological preparations of antigens using anti-*P. insidisosum* antibodies has been reported in the form of immunofluorescence (FA) [17••] and immunoperoxidase (IP) formats [46]. Using these methodologies, the detection of *P. insidiosum* hyphal elements in histological preparations is highly sensitive and specific [15, 17••, 46]. Unfortunately, these are in-house methodologies and there is no standardization of the described techniques.

DNA Testing The use of molecular tools has been implemented as a diagnostic support in the absence of culture [14••, 40, 47•]. The extraction of total genomic DNA from clinical samples and the use of putative specific primers from in-house studies has been proven to be of help in the preliminary identification of this pathogen. However, the detection of PCR amplicons by itself has to be evaluated with caution, since the specificity of those primers is still under investigation [48]. Thus, putative *P. insidiosum* PCR amplicons have to be properly sequenced to confirm their identity.

Culture Culture is the gold standard test, but it is insensitive, and thus false negatives are common [17••]. The collected clinical samples have to be transported in water at room temperature in 24 h or less. The biopsied tissue (cut in 5 mm pieces) and/or deep scrapings are placed into 2.0 % Sabouraud dextrose plates and incubated at 37 °C and room temperature. At 37 °C, *P. insidiosum* develops very rapidly. Glabrous submerged radiate white/yellowish colonies are usually detected in less than 24 h (Fig. 2). Microscopically, sparsely septate hyphae 3 to 10 μm without fruiting bodies are found [15, 17••]. The proper identification of putative cultures is done by the formation of biflagellated zoospores using the Mendoza and Prendas [49] technique and by DNA sequencing analysis (Fig. 2) [17••]. More recently, the use of 30 μg minocycline disks has been mentioned as an aid to identify *P. insidosum* in culture [50]. However, with the introduction of yet another oomycete pathogenic for mammals, this technique has to be evaluated on strains of *Lagenidium* species as well. The technique may be difficult to interpret, especially if Lagenidium is also susceptible to this test (see below).

Treatment of the Infections Caused by Pythium insidiosum

As was mentioned earlier in this review, these oomycetes are intrinsically resistant to most antifungal drugs (see above) [51]. Thus, in the past 40 years, contradictory results have been published on the efficacy of these drugs in different hosts with the disease [17••]. Despite in vitro testing of numerous novel antifungal drugs with potential inhibitory capabilities, the in vivo efficacy of such drugs evaluated in animal models and in cases of pythiosis proved to be disappointing [52, 53]. Thus, for a long time, the treatment of choice in humans and other animals with pythiosis has been surgery and iodides [17••]. However, the high recurrence rate in surgically treated patients and the toxic side effects of iodides are significant limitations.

In 1981, Miller [54] noted that the proteinaceous immunogens extracted from in vitro cultures of *P. insidiosum* had the capacity to cure some cases of horse pythiosis with cutaneous infection. Mendoza et al. [45] later confirmed such observations, and prepared a new formulation that had enhanced curative properties when injected in hosts with cutaneous and arterial pythiosis [36•, 39•]. Apparently,

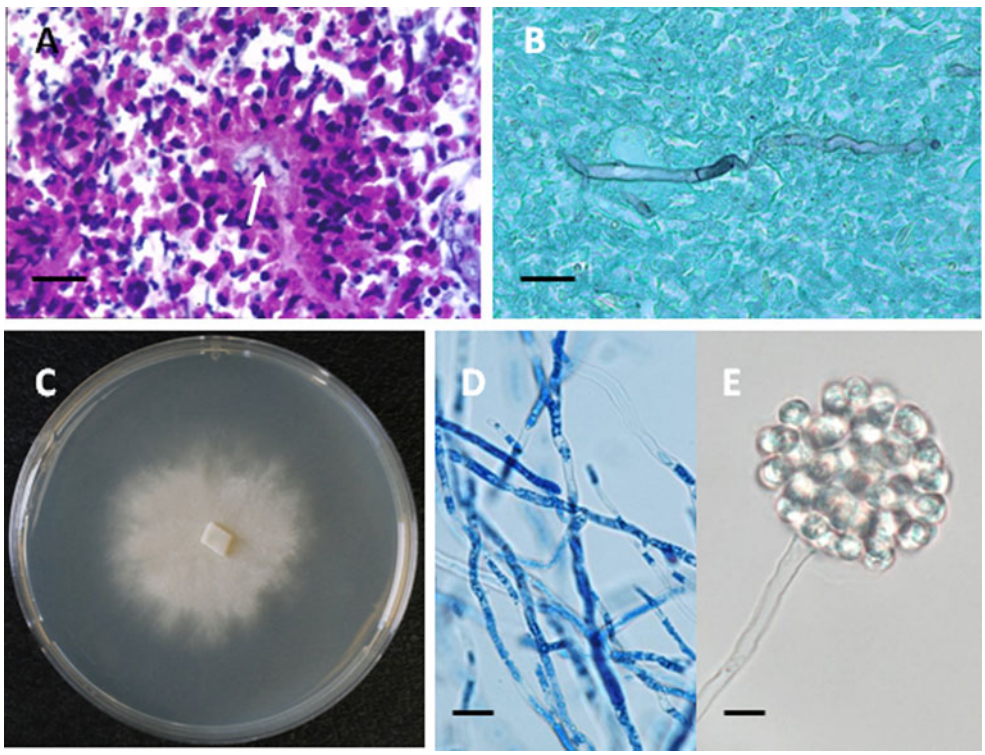

Fig. 2 Panel **a** depicts a histological tissue section of a horse with pythiosis stained with Hematoxylin & Eosin (H&E) (*Bar*=40 μm). Note the presence of *Pythium insidiosum* hyphae (*white arrow*). In Panel **a**, the eosinophils degranulate around *P. insidiosum* hyphae, forming an eosinophilic precipitate known as the Splendore/Hoeppli phenomenon. Panel **b** (*Bar*=30 μm) shows a silver stained hypha of *P. insidiosum*. The presence of an occasional septum can be detected in long structures, such as the one in this figure. *P. insidiosum* on 2 % dextrose agar (Sabouraud) develops submerged white/yellowish glabrous colonies (Panel **c**). On Sabouraud dextrose agar, *P. insidiosum* develops sparsely hyphae lacking fruiting bodies (Panel **d**, *Bar*=25 μm). Panel **e** (*Bar*=25 μm) shows a single *Lagenidium* sp. sporangium containing numerous biflagellate zoospores ready to hatch

immunotherapy works by presenting the infected host with *P. insidiosum* immunogens in a different fashion than during natural infection [36•]. Different groups had concluded that immunotherapy seems to switch key interleukins from a Th2 (infection) to a Th1 (protection) response [36•, 55]. These studies claimed that such switching also triggers the release of activated macrophages, cytotoxic T lymphocytes and natural killer cells that seem to eliminate the pathogen from the infected tissues. Because the curative rate of immunotherapy is about 55 % in humans and dogs, and 70–80 % in horses and cattle [36•], currently, a combination of surgery, antifungal drugs (terbinafine) and immunotherapy has been implemented, with a dramatic increase in cure rate [55, 56].

Lagenidium spp.

Infections attributable to a new mammalian pathogenic oomycete species were first diagnosed in dogs sometime in 1999, although it was officially reported in 2000 simultaneously in two separate publications [14••, 15]. In these reports, the authors introduce the name oomycosis to label the infections caused by *Lagenidium* spp. that affect dogs. The term oomycosis was later changed to lagenidiosis, to reflect the name of the etiologic agent of this emerging disease, which was apparently caused by a novel oomycete species. Since then, several new cases of lagenidiosis in cats, dogs, and humans have been mentioned [57, 58]. It was soon apparent that at least two different *Lagenidium* species were involved in cases of mammalian lagenidiosis [58]. The proposed species were termed *L. caninum* and *L. karingii*, but an official description of these species has yet to be presented. More recently, several cases of dogs and a cat with lagenidiosis were diagnosed at Michigan State University, confirming the original descriptions of Grooters [14••] and Grooters et al. [15] [Mendoza & Vilela, personal communication].

Geographical Distribution, Epidemiology and Pathogenesis

So far, the infections caused by *Lagenidium* spp. have been diagnosed in cats, dogs, and humans inhabiting the southern United States, with some cases also described in northern States [14••, 15, 57–59]. A recent human case of lagenidiosis in a Thai human patient with keratitis [60] suggests that the location of *Lagenidium* spp. pathogenic for mammals in the environment may follow the same geographical distribution as the cases recorded for *P. insidiosum* [17••, 48]. Therefore, it does not come as a surprise that the first cases of dog lagenidiosis occurred in animals that entered putative contaminated ponds, as reported in dog pythiosis cases [14••, 24]. Because *P. insidiosum* develops zoospores as a part of its life cycle in nature, it has been suggested that *Lagenidium* species may use a natural substrate (perhaps plants) to also complete their life cycle in nature [14••, 15, 49]. Thus, it is quite possible that the same mechanisms of infection described for *P. insidiosum* are also valid for *Lagenidium* spp. [36•]. Under this scenario, when a mammalian host with a skin injury enters a contaminated pond, the zoospores of *Lagenidium* will encyst upon contact with the injured skin and mechanically penetrate the tissue, causing lagenidiosis. In the initial descriptions of the disease, Grooters [14••] and Grooters et al. [15] showed that the inflammatory response in dogs with lagenidosis was strikingly similar to that encountered in cases of dog pythiosis. Based on these reports, we believe that similar mechanisms of pathogenesis are also triggered during infections caused by the mammalian pathogenic *Lagenidium* spp. (see *P. insidiosum* pathogenesis) [9••, 36•].

Grooters [14••] and Grooters et al. [15] called attention to the similarities between the 18S SSU DNA of *Lagenidium* spp. recovered from dogs and *L. giganteum*, a pathogen of mosquito larvae. The authors also mentioned that *L. giganteum* had been used as a biological control in the southern USA, and was approved by the Environmental Protection Agency (EPA) [14••, 57, 58]. Although *L. giganteum* poorly develops at 37 °C after 5 days of incubation, *Lagenidum* sp. from dogs (also named as *L. caninum* by Grooters) grows in less than 24 h. Based on this information, we can speculate that the remarkable morphological and DNA similarities between these two pathogenic oomycetes are of concern, since the tools to separate them are currently lacking. In addition, the original investigators claimed that they could not reproduce experimental infection after inoculation of *L. giganteum* in mammals [61]. However, *P. insidiosum* cannot be experimentally reproduced in mammals other than rabbits [17••, 35]. As far as we know, no one has evaluated *Lagenidium* sp. experimentally in rabbits.

Clinical and Pathological Features

The clinical manifestations of lagenidiosis in dogs include the development of small ulcerate tissues that rapidly enlarge, and more often develop subcutaneous multicentric lesions [14••, 15, 59]. As in cases of dog pythiosis, the lesions are, firm, nodular, with sinus tracts, and ulcerate. These types of lesions are consistently misdiagnosed as bacterial infections and initially treated with antibiotics. Lymphadenopathies and edematous swelling around the infected areas are common [15]. According to Grooters [14••], the cutaneous and subcutaneous lesions by *Lagenidium* sp. are frequently progressive, and do not respond to the therapies used for pythiosis, including immunotherapy [unpublished data of the authors]. Intestinal infection in dogs with *Lagenidium* sp. is unusual, but dissemination of the pathogen to large vessels, pulmonary

hilus, and mediastinum has been mentioned [15]. A case of lagenidiosis in a North Carolina cat was studied by the authors [unpublished data]. The cat lesions were protracted and limited to subcutaneous tissues. Ulcerate tissues or sinus tracts were not observed (Fig. 3). In contrast, four cases of subcutaneous dog pythiosis investigated in our laboratory [unpublished data] were characterized by the development of multicentric ulcerated lesions with sinus tracts and lynphadenopathies in several parts of their anatomy (Fig. 3). More recently, a Thai human case was reported, from which a strain, identified as *Lagenidium* sp. by sequencing analysis, was described [60]. The authors stated that clinical picture of the case could not be differentiated from cases of fungal infections or keratitis caused by *P. insidiosum*.

The histopathology of *Lagenidium* spp. infection is very similar to that reported by the pathogens *P. insidiosum*, and two entomophthoromycetous fungi, *Conidiobolus* spp. and *Basidiobolus* spp. [14••, 15, 62]. In brief, an eosinophilic granulomatous inflammation of the affected skin and subcutaneous tissues is common. Areas of necrosis and micro-abscesses containing numerous eosinophils, mast cells, neutrophils, and giant cells are also observed (Fig. 4). Within the micro-abscesses, hyaline ribbon-type

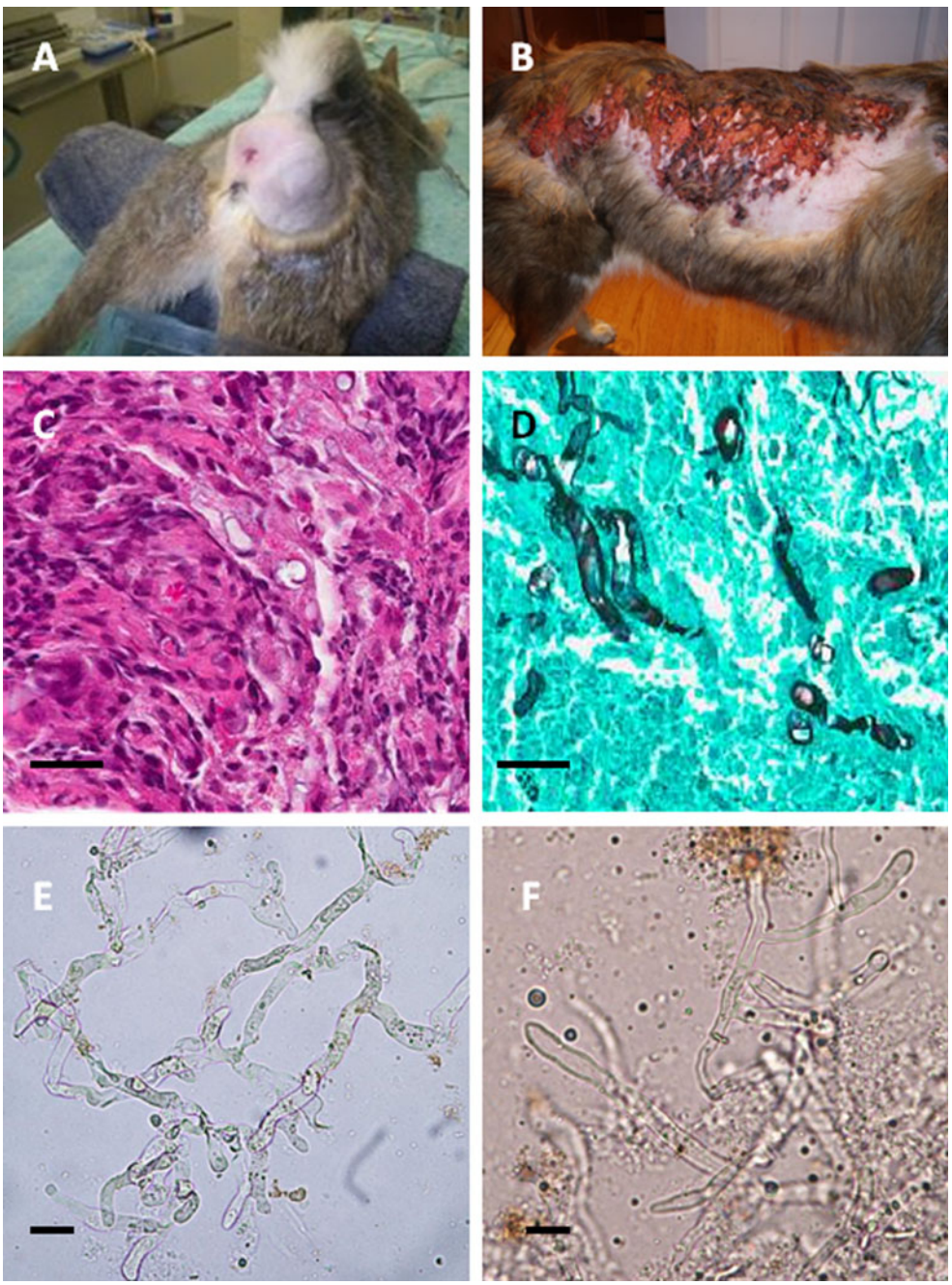

Fig. 3 Panel **a** shows protracted subcutaneous granulomatous lesions without ulcerate tissue caused by *Lagenidium* spp.in a North Carolina cat, very similar to that reported in cases of cat pythiosis. In contrast, Panel **b** shows a dog with disseminated multicentric lesions in cutaneous and subcutaneous tissues caused by *Lagenidium* spp. Panels **c** and **d** show the hyphal elements of *Lagenidium* species in two histological preparations from a case of dog lagenidiosis. Note in panel **c** (*Bar*=35 μm) the ghosts of empty hyphal elements of *Lagenidium* sp. (*arrows*). Panel **d** (*Bar*=35 μm) depicts the presence of several silver stained hyphae of *Lagenidium* sp. from Panel **c**. Panels **e** (*Bar*=30 μm) and F (*Bar*=30 μm) depict ribbon type hyphae in 10 % KOH wet mount preparations from two different cases of dog lagenidiosis. The presence of sparsely septate hyphae is the main feature of *Lagenidium* spp. pathogenic for dogs

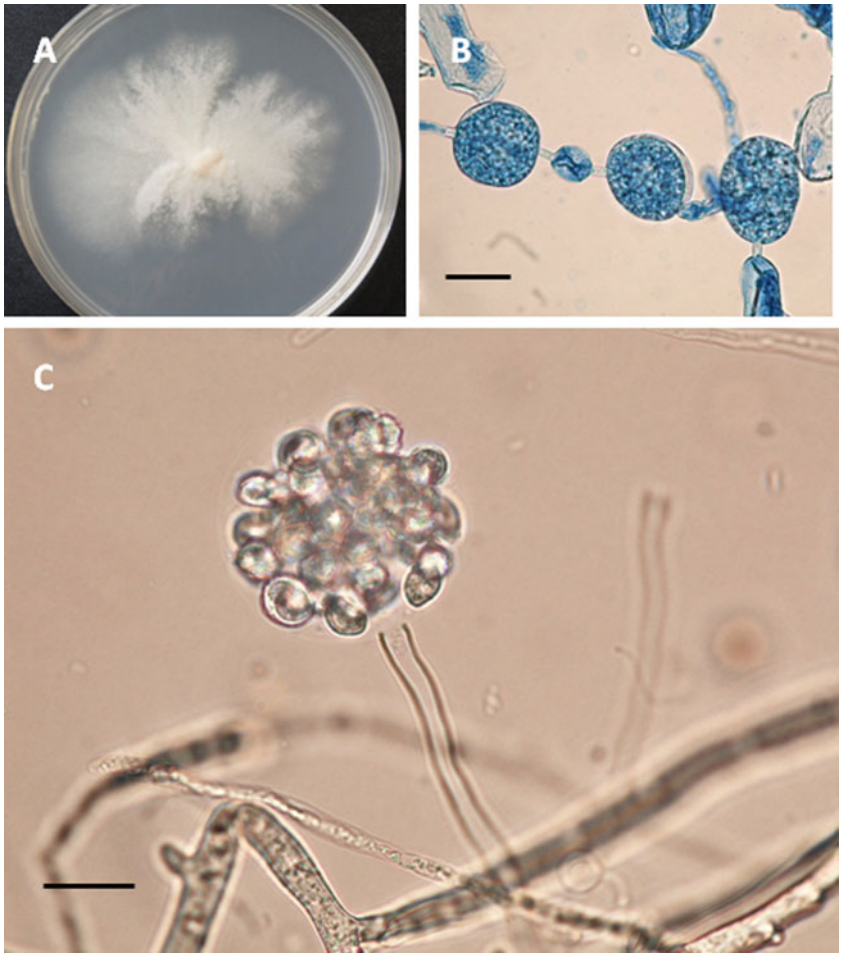

Fig. 4 Panel **a** shows a culture on 2 % dextrose agar plate of *Lagenidium* sp. recovered from a dog with lagenidiosis. *Lagenidium* spp. develops submerged white/yellowish glabrous colonies that sometimes demonstrate furrows at the center of the colonies. The morphological and taxonomic features of *Lagenidium* spp. in cultures have yet to be resolved. Panel **b** shows spherical structures in chain connected by short pieces of hyphae (*Bar*=45 μm). Broad hyphae with few septa are also present. Panel **c** (*Bar*=45 μm) shows a single sporangium of *Lagenidium* sp. from Panel **a**. Note the ribbon type hyphae as well of the size of the biflagellate zoospores inside the sporangium

hyphal elements of *Lagenidium* sp. can be found. The hyphae are difficult to visualize with H&E staining, and sometimes appear as transverse sparsely septate or round (sagittal cuts) empty ghost hyphae structures. In H&E, eosinophilic material surrounding the entire hyphal structures (Splendore/Hoeppli phenomenon) is present within micro-abscesses [14••, 15, 21]. In silver stained samples, the hyphae measure 6 to ≤20 μm in diameter, much bigger than *P. insidiosum* hyphae (4 to 10 μm), but very similar in size to *Conidiobolus* and *Basidiobolus* spp. [21, 62]. The finding of ribbon-type hyphal elements in Giemsa cytological preparations from cases of dog lagenidiosis has been reported [59].

Laboratory Diagnosis

The differential diagnosis between the oomycete *Lagenidium* spp. and the entomophthoromycetous fungi *Conidiobolus* sp. and *Basidiobolus* sp. can be made by culture. The latter two fungi develop typical conidia and/or zygospores that facilitate their identification [62]. Although the diameter of hyphae may be an important clue to separate *P. insidiosum* from *Lagenidium* spp., not only in the infected tissues but in cultured samples, an accurate identification of these two oomycetes is only possible with the use of molecular assays (see below) [47•, 62].

Wet Mounts and Stains The hyphal elements of *Lagenidium* species can be detected from biopsied clinical samples collected in the infected areas, preferably from the advancing areas of the ulcers. The sample has to be cut in 5 mm blocks and placed in 10 % KOH (Fig. 4). Scrapings from the borders of the ulcer can be also prepared for cytological evaluation by staining with Giemsa [59]. Broad branched hyphal structures are usually visualized with either of these two methodologies.

Serological Assays Serological assays to detect *Lagenidium* spp. infections in humans and animals have been developed [14••, 15]. An early WB assays showed that antibodies in sera from dogs infected with *Lagenidium* spp successfully detected the antigens of *Lagenidium* spp. [15]. However, this test showed random patterns that were difficult to interpret. In ELISA, antibodies in the sera of dogs with lagenidiosis showed strong cross-reaction to *P. insidiosum* antigens, a finding that limited its potential use for diagnostic purposes

[14••, 15]. Despite this limitation, ELISA has been suggested for monitoring the response to treatment in cases of dog lagenidiosis [14••]. An imunohistochemistry assay to detect *Lagenidium* spp. in infected tissues has been mentioned, but is not yet available [14••].

DNA Testing PCR-based assays have been developed for both *P. insidiosum* (see above) and *Lagenidium* spp. [47•], and have been successfully tested in the identification of putative *Lagenidium* spp. cultures, biopsied tissue containing broad *Lagenidium*-like hyphae, and paraffin-embedded tissue from suspected cases of lagenidiosis [47•]. The PCR-based assay accurately differentiated between *Lagenidium* spp. hyphal elements and the structures developed by *P. insidiosum* in culture or infected tissue [47•]. In this assay, *P. insidosum* produces a 105 bp amplicon, whereas *Lagenidium* spp. demonstrates a 76 bp product. Although the strains of *Lagenidium* that are pathogenic for dogs have yet to be properly described, molecular tools have showed the presence of at least two different species of *Lagenidium* that are pathogenic for mammals [57, 58]. Grooters [58] provisionally named them as *L. caninum* and *L. karlingii*.

Culture As for cases of pythiosis, clinical samples sent to the laboratory to investigate putative cases of lagenidiosis have to be submitted within the first hour of collection. If the sample has to be submitted to a reference laboratory, the specimens have to be transported in water at room temperature within 24 h. The morphological features in culture of the colonies developed by *Lagenidium* spp. pathogenic for dogs and a cat (unpublished data) showed different phenotypic attributes. For instance, two cases of dog lagenidiosis studied in our lab showed submerged flat glabrous white/yellowish colonies (Fig. 4). Microscopically, broad 8 to 20 μm coenocytic hyphae with spherical structures 40 to 80 μm in diameter connected by short pieces of hyphae with characteristic septa between them were found (Fig. 4). In contrast, in a case from a cat with lagenidiosis, the presence of long elongated structures attached to coenocytic hyphae was the main feature. The morphological features of the spherical structures of the *Lagenidium* spp. recovered from dogs, can be used to differentiate these strains from those of *P. insidiosum* in culture [17••].

Management

Early diagnosis of lagenidiosis is essential for appropriate treatment of the infection [14••, 15]. The mammalian pathogenic oomycetes respond poorly to most antifungals (see above) and also to surgery, and *Lagenidium* spp is not an exception [14••]. In cases of lagenidiosis, the management of choice is always radical surgery. However, this strategy is not always possible, due to the advanced stages of the disease by the time an accurate diagnosis is made [15]. *P. insidiosum*-immunotherapy has proven to be ineffective, even using *Lagenidium* immunogens extracted from strains recovered from cases of dog lagenidiosis (unpublished data). A Thai case of keratitis caused by *Lagenidium* sp. was successfully treated with repeated penetrating keratoplasty [60]. The in vitro evaluation of traditional and new antifungal drugs on strains of *Lagenidium* spp. was recently reported, but the results were not tested in vivo in experimentally infected animals.

Conclusion

Infections caused by the oomycetes *Pythium insidiosum* and *Lagenidium* species are considered emerging fungal-like (parafungal) diseases. The fact that few physicians and clinical laboratory workers are familiar with these emerging oomycetes is of concern. Thus, these etiologies should be included in medical, veterinary and clinical laboratory sciences curricula. Moreover, the phenotypic similarities between the mammalian pathogenic oomycetes and some filamentous fungi without septa (zygomycetes) requires new laboratory tools to properly differentiate these two groups of pathogens [63]. This is essential for selection of appropriate management, since most antifungal drugs have poor activity against the mammalian pathogenic oomycetes [52, 53]. We have reviewed the most relevant aspects of these mammalian pathogenic organisms with the intention of introducing their major aspects and characteristics.

Compliance with Ethics Guidelines

Conflict of Interest Leonel Mendoza receives a salary from Michigan State University.

Raquel Vilela declares that she has no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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