Pythium insidiosum

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Pythium insidiosum

LEONEL MENDOZA

P135.1 Members of the oomycetous genus *Pythium* are ecologically and physiologically unique. They occur in soil and aquatic habitats worldwide. *Pythium* species are important plant pathogens causing seed decay, pre-emergent and post-emergent damping off, root rot of seedlings, and rot of stored foodstuffs. In addition, some species of *Pythium* have been reported to cause disease in fish. *Pythium insidiosum*, however, is the only member of the genus that has been recognized as a mammalian pathogen. Chandler et al. (1980) coined the term pythiosis to include all clinical and pathological manifestations caused by *P. insidiosum* in mammals. In horses, the disease has been long known as bursatee, leeches, granular dermatitis, hyphomycosis destruens equi, phycomycosis, espundia, summer sores, and swamp cancer. Pythiosis in mammals is characterized by the development of cutaneous, subcutaneous, blood vessel, and intestinal lesions and, less frequently, by the involvement of bones and lungs. If not treated, the disease progresses rapidly, becoming life-threatening.

H135.1 ## HISTORY

P135.2 The first reported cases of equine pythiosis were described in the middle of the last century when veterinarians studied cutaneous granulomas among equines in India. The etiology of the disease was not established.

The first well-documented cases, in which the 'fungal-like' nature of the infection was suggested, were those published by Smith (1884), Fish (1895–96), and Drouin (1896). In Indonesia, de Haan and Hoogkamer (1901), working with several equines with cutaneous granulomas, isolated the etiologic agent of pythiosis for the first time. Their isolate, however, could not be identified because it did not sporulate on their media. These investigators named the disease hyphomycosis destruens. This name was modified later by de Haan (1902) to hyphomycosis destruens equi. In 1924 another Dutch scientist, working with horses in Indonesia, completed the most comprehensive papers on equine pythiosis ever published (Witkamp 1924, 1925). He also isolated a nonsporulating organism from equine cutaneous granulomas and covered several aspects of the disease, including its clinical signs, pathology, microbiology, animal inoculation, diagnosis, treatment, and immunology. Unfortunately, this classic work remained largely unnoticed, in part because of its Dutch language, but also because of the contemporaneous finding that cutaneous granulomas in horses may also be caused by nematode species of the genus *Habronema* (Ransom 1911). Thus, the terms used to describe pythiosis were also indiscriminately used for equine cutaneous habronemiasis.

P135.3 The early findings on the 'fungal' nature of cutaneous granulomas in equines were obscured by the new

hypothesis of equine habronemiasis, and the presumed mycotic etiology was forgotten. Thirty-seven years later, Bridges and Emmons (1961) isolated a sterile, filamentous microorganism from equine cases in Texas and Florida, similar to those that had been studied by de Haan and Hoogkamer and Witkamp earlier in the century. They believed that they had isolated 'a species of *Mortierella*' – a zygomycete. The filamentous isolate was named *Hyphomyces destruens*, based on the disease name previously coined by de Haan and Hoogkamer (1901, 1903). It was not clear, however, if the authors intended to introduce a new binomial for the etiologic agent of cutaneous granulomas in horses. In 1974, Austwick and Copland reported that an organism, isolated from horses in Papua New Guinea afflicted with swamp cancer, formed biflagellate zoospores after transfer to a petri dish of sterile water to which a sterilized decayed piece of rotten maize silage had been added. They concluded that the organism should be classified in the oomycete genus *Pythium* but they failed to provide a scientific name for their isolate.

P135.4   Later, Ichitani and Amemiya (1980) isolated a filamentous microorganism from a Japanese horse with granular dermatitis. Based on the production of smooth oogonia and aplerotic oospores, they identified their isolate as *Pythium gracile*. In 1987, de Cock et al., working in Costa Rica with numerous isolates of a *Pythium* sp., recovered from horses that had espundia (a regional name used for pythiosis), concluded that these isolates belonged to an undescribed species of *Pythium* and the binomial *P. insidiosum* was validly introduced. They also reported that other isolates from cows, dogs, horses, and humans, including the one described as *P. gracile*, also belonged to the single species *P. insidiosum*. Almost concurrently an isolate, from horses in Australia, was also described as a new species and named *Pythium destruens* by Shipton in 1987. Later this isolate was studied by Mendoza and Marin (1989) and it was found to be indistinguishable from the strains isolated from other pythiosis cases; it thus became a synonym for *P. insidiosum*. Recently, Schurko et al. (2003a,b), using molecular tools, confirmed that *P. insidiosum* is the only etiologic agent in the genus causing pythiosis in mammals.

P135.5   Since 1961 several new cases of pythiosis have been reported in equines (Habbinga 1967; Hutchins and Johnston 1972; Connole 1973; McMullan et al. 1977; Murray et al. 1978; Miller and Campbell 1982b; Mendoza and Alfaro 1986), cats (Bissonnette et al. 1991; Thomas and Lewis 1998), cattle (Miller et al. 1985; Santurio et al. 1998), dogs (Pavletic et al. 1983; Foil et al. 1984; Thomas and Lewis 1998; Graham et al. 2000), humans (Thianprasit 1986, 1990; Rinaldi et al. 1989; Sathapatayavongs et al. 1989; Chetchotisakd et al. 1992; Triscott et al. 1993; Virgile et al. 1993; Imwidthaya 1994b), and in captive bears and a camel in South

Carolina and Florida zoos respectively (personal communications, L. Kaufman and A.A. Padhye, and J.F.X. Wellehan).

TAXONOMY AND MORPHOLOGICAL FEATURES OF *PYTHIUM INSIDIOSUM*

Pythium insidiosum is an organism classified in the   P135.6 kingdom Straminipila, class Oomycetes, order Pythiales, and family *Pythiaceae* (Dick 2001). Although Dick (2001) suggests the term Peronosporomycetes to rename the class Oomycetes in the kingdom Striminipila, for convenience, we will continue using the term Oomycetes throughout this chapter. This was motivated also by his position to treat the straminipilans as members of the kingdom Fungi. In recent years, however, strong phylogenetic evidence has been generated indicating that the straminipilans are not fungi, but protistal microbes very close related to algae and plants (Herr et al. 1999; Baldauf et al. 2000). Patterson in 1989 proposed the term Stramenopila. This name has been widely used. However, Dick (2001) recently modified it to Straminipila. He stated that the term Stramenopila is a bad derivation from the latin 'stramini'= a straw and 'pilus'= a hair. So, the straminipilans are organisms bearing tubular tripartite hairs. Therefore, the right spelling of this term should be Straminipila. Dick (2001) also indicated that the spelling 'straminopile,' appearing in the *Dictionary of fungi* (Hawksworth et al. 1995), was an unintentional misprint corrected in the 2001 edition of the same dictionary (Kirk et al. 2001).

In culture, *P. insidiosum* develops fungal-like sparsely   P135.7 septate hyphae; thus it erroneously has been referred to as a microorganism belonging to the kingdom Fungi. All of its phylogenetic features, however, correlate with the kingdom Straminipila (Baldauf et al. 2000; Martin 2000; Dick 2001). On this basis, *P. insidiosum* should only be referred to as a protist or a parafungal organism. As with other oomycetes, *P. insidiosum* grows relatively well on a variety of media. On cornmeal agar, colonies are colorless to white and submerged, with short aerial filaments, and a finely radiate pattern. The hyphae range between 4 and 10 μm in diameter with perpendicular lateral branches. Cross-septa are only occasionally observed in young hyphae, but they are abundant in old viable hyphae. Appressoria and hyphal swellings, measuring 12–28 μm in diameter, are common in laboratory cultures (Figure 24.1a, b).

Production of zoosporangia is only possible in water   P135.8 cultures incubated at 28–37°C. Zoosporangia increase in number when *P. insidiosum* is placed in contact with pieces of grass leaves in water containing a minimal quantity of different ions (including Ca^{2+}) (Mendoza and Prendas 1988). Early-stage sporangia cannot be differentiated from vegetative hyphae. At maturity, sporangial protoplasm flows into a discharge tube and
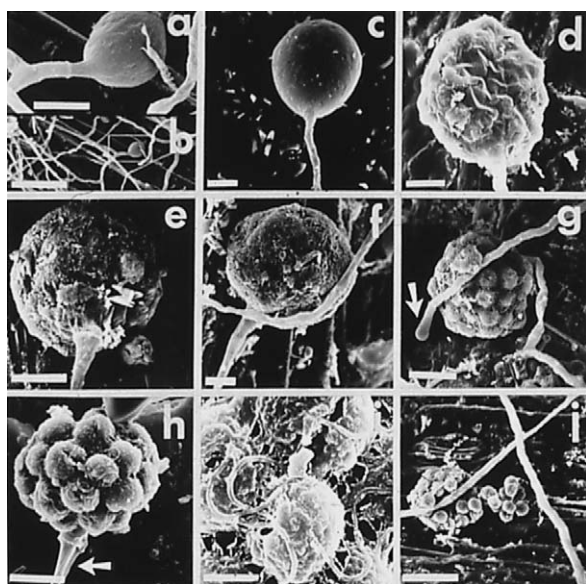
Figure 24.1 *Transmission electron microscopy of* Pythium insidiosum*'s different stages of development. The formation of globose vesicles and hyphae on a leaf are the first steps in the colonization of a plant host (a, b). The electron photographs show sporangial formation leading to the release of secondary-type zoospores (c–j). Bar:* **(a, c)** *28 μm,* **(b)** *120 μm,* **(d)** *20 μm,* **(e)** *30 μm,* **(f)** *15 μm,* **(g)** *32 μm,* **(h)** *18 μm,* **(i)** *15 μm,* **(j)** *50 μm. (Reproduced, with permission, from Mendoza et al. 1993)*

Figure 24.2 *Transmission electron microscopy of* Pythium insidiosum*'s motile zoospores (a–c). Encysted zoospores are characterized by their globose morphology (d–f). Arrows indicate the place once occupied by the flagella. An amorphous substance used to bind the zoospores to the host's tissue is evident over the surface of encysted zoospores. Zoospores that encyst away from leaf or animal tissues do not secrete this amorphous material* **(f)**. *Bars:* **(a, b)** *9 μm,* **(c, d, f)** *10 μm,* **(e)** *5 μm. (Reproduced, with permission, from Mendoza et al. 1993)*

forms a vesicle. It is globose and hyaline, and measures 20–60 μm in diameter. Through progressive cleavage, biflagellate zoospores are formed inside the vesicle (Figure 24.1c). The internal developmental process within the undifferentiated vesicle and the release of zoospores takes about 35 minutes. The zoospores mechanically break the vesicle's wall and upon emergence swim for about 20 minutes and then encyst (Figure 24.1j). The zoospores are reniform and of the secondary biflagellate type. The two flagella arise from the deeper part of a lateral groove and seem to emerge from a common point. They are unequal in length. The anterior shorter flagellum is of the tinsel type and is covered with mastigonemas (small hair-like structures). The posterior flagellum is of the whiplash type and lacks mastigonemas (Figure 24.2a–c). After encystment, the zoospore's flagella are detached and it becomes spherical. When chemotactically stimulated by a suitable substrate (plant or animal tissue), the zoospores secrete anamorphous sticky material which covers their surface, and they become attached to the plant's or animal's surface. This substance, which may be a glycoprotein, has been implicated as a potential virulence factor in establishing infection (Mendoza et al. 1993) (Figure 24.2d–f).

P135.9 In contrast to other species of the genus, only a few strains of *P. insidiosum* have been reported to produce oogonia. Thus, the mechanism implicated in the process of oogonial formation in *P. insidiosum* remains obscure.
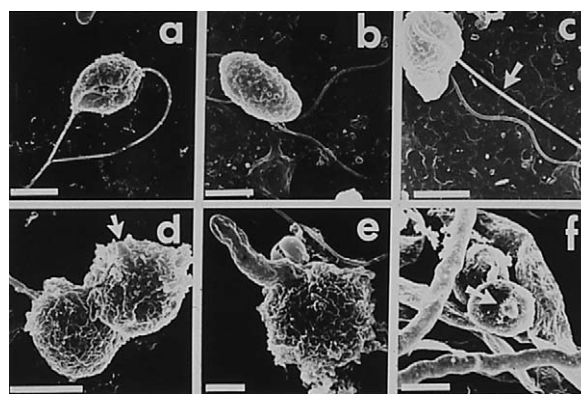
When oogonia develop in vitro, they are intercalary, smooth, and subglobose. They have a rigid fertilization tube measuring 23–30 μm in diameter and may have one to three declinous antheridia per oogonium. The antheridia are attached over their entire length to the oogonium (de Cock et al. 1987; Shipton 1987). The oospores are aplerotic, or almost plerotic, and are pressed to one side of the oogonium by the rigid fertilization tube. They measure 20–25 μm in diameter (Figure 24.3k) (de Cock et al. 1987; Shipton 1987). The morphology of the mature oogonia is the basis for speciating members of the genus *Pythium*.

P135.10 Schurcko et al. (2003a,b) using phylogenetic tools, reported that in the analyses done on 23 isolates of *P. insidiosum* from the Americas, Asia, and Australia, all clustered together according to their geographical areas (Figure 24.4). This study showed that *P. insidiosum* is more closely related to each other than to any other *Pythium* species, and quite different from the genera *Phytophthora* and *Lagenidium*. Interestingly, the third cluster of *P. insidiosum* strains was made of isolates from Thailand and the USA. Schurcko et al. (2003b) suggested that these unique isolates could belong to new species very close related to *P. insidiosum*. However, they did not treat these cryptic variants as such.

P135.11 Recently, Grooters (2003) found that clinical cases initially thought to be pythiosis in dogs were not caused by *P. insidiosum*, but by a new species in the genus *Lagenidium*. She based her findings on morphological and molecular studies. In a recent phylogenetic study, however, Schurko et al (2003a,b) described at least three cryptic strains of *P. insidiosum*. These strains formed a sister clade with the other 20 *P. insidiosum* isolates studied by them. They stated that all *P. insidiosum*, and the other *Pythium* spp, were closer to the genus

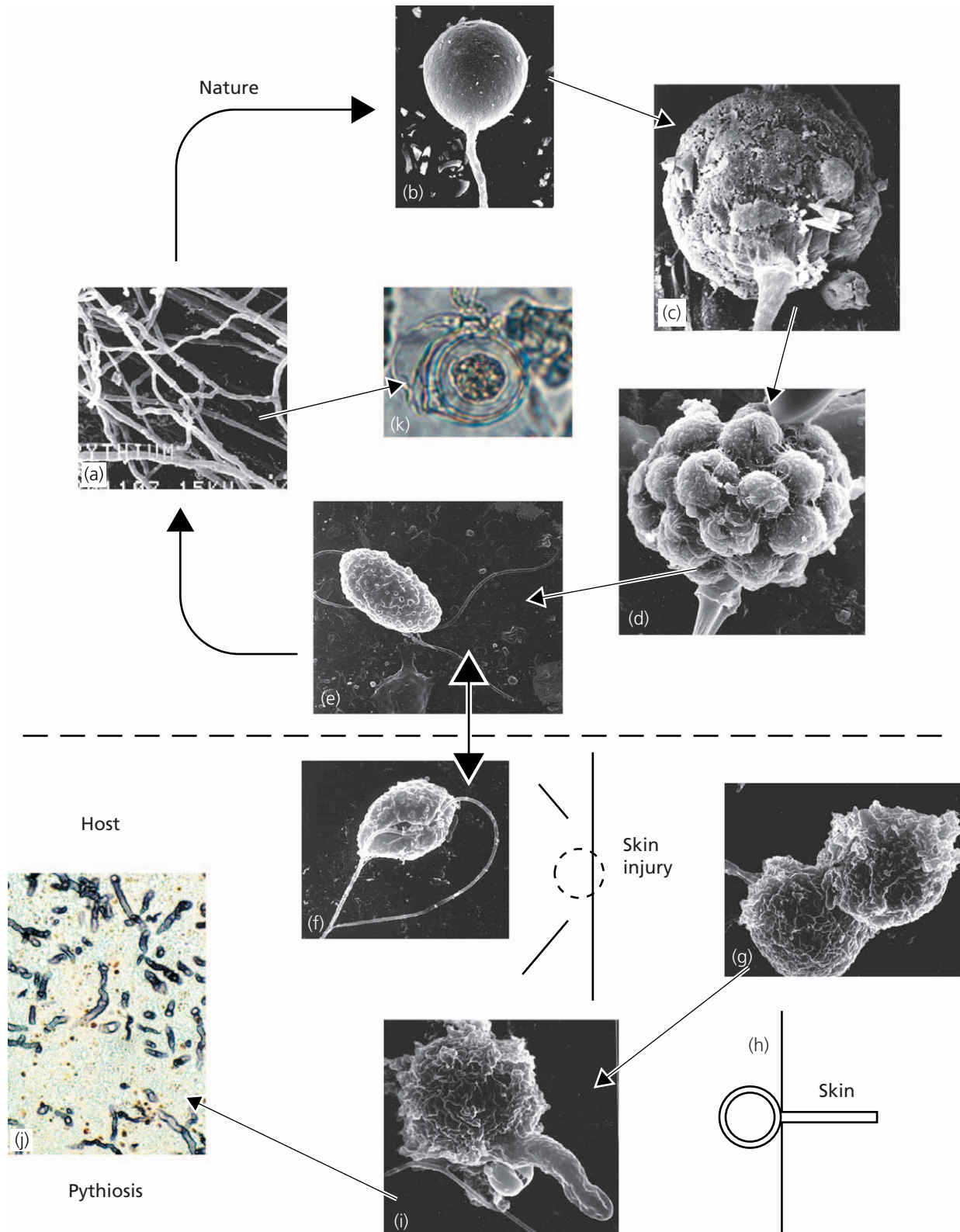FIG135.3 **Figure 24.3** *(a–e, k, in upper panel) The life cycle of* Pythium insidiosum *in nature. Plant tissue is first colonized by* **(a)** *hyphae of* P. insidiosum, *and then* **(b–d)** *the differentiation of sporangium into mature stages leads to* **(e)** *zoospore release.* **(f)** *The zoospores swim to locate another plant or may be attracted by injured animal tissue. The encysted zoospores were (g, dots) attached to tissue by a sticky substance, germinate* **(h)**, *invade the host* **(i)**, *and cause pythiosis.* **(j)** *The formation of masses, called kunkers, occurs only in horses. The production of oospores occurs in nature and they may serve as* **(k)** *resistant spores.*
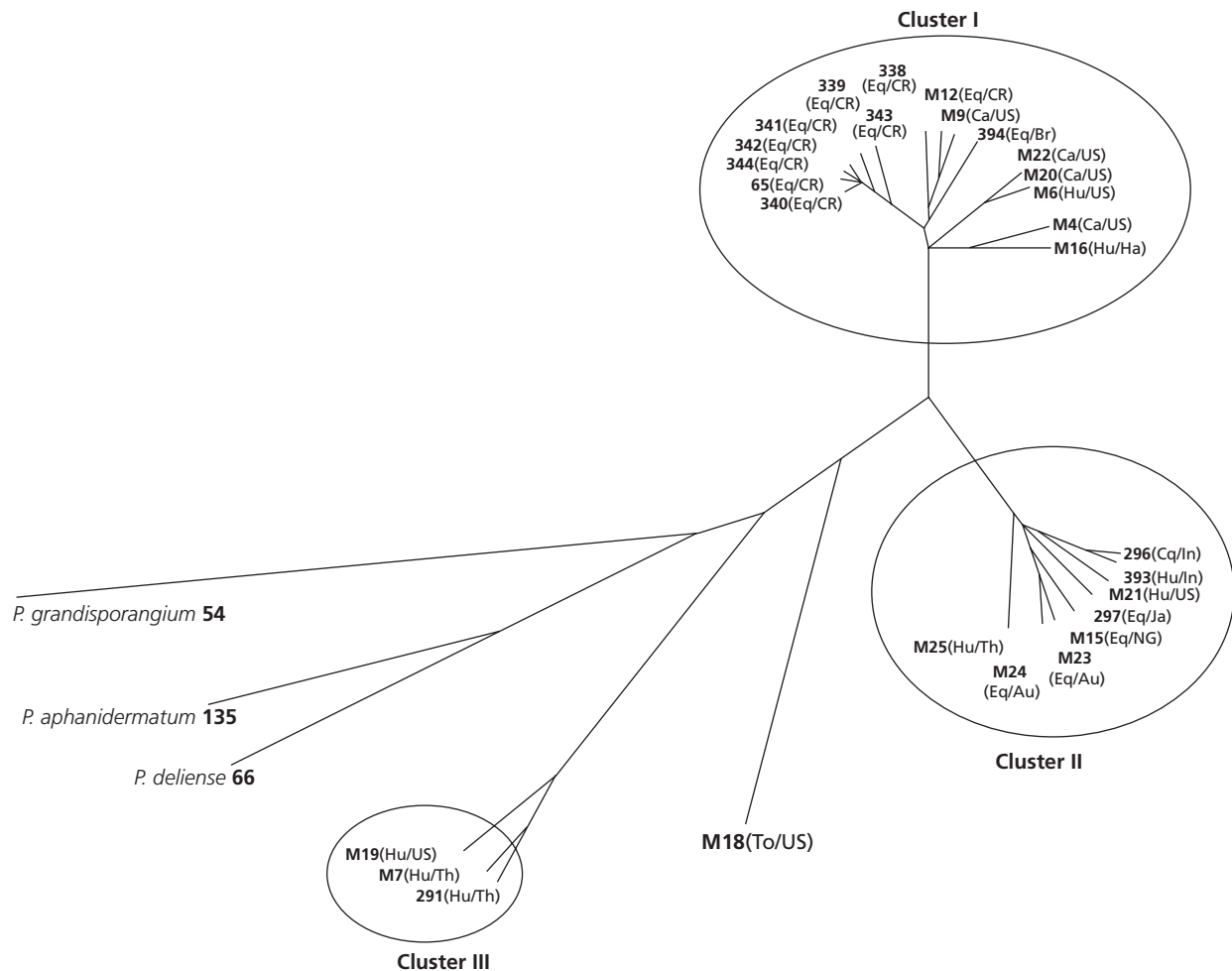
Figure 24.4 *The phylogenetic tree shows an unrooted unweighted pair-group method average (UPGMA) phenogram depicting the clusters formed by the 23* Pythium insidiosum *isolates investigated by Schurko el at. (2003a). The numbers and letters in bold identified the strains used to build the tree and in parenthesis the origin and geographical location of the strains. Cluster I contains all the strains from the Americas, cluster II, strains from Asia and cluster III (including M18) has a mixture of Asian and American isolates. (Reproduced, with permission, from Schurko et al. 2003a)*

Lagenidium and far away from the genus *Phytophthora*. Thus, it could be that the strains studied by Grooters (2003) may be related to the third group described by Schurko et al (2003a,b) rather than being new members of the genus *Lagenidium*. However, this conjecture needs verification.

EPIDEMIOLOGY

Pythium spp. are ecologically versatile microorganisms. They occur in practically all soils and wet environments. They are among the most destructive phytopathogens, inflicting economic losses on a wide variety of crops. However, of all the described species (about 120) only one, *P. insidiosum*, has been implicated as an etiologic agent of mammalian disease. Since the last century, water has been related to cases of cutaneous granulomas in equines. It was noted that, after horses had grazed for a lengthy period in stagnant water, they frequently developed pythiosis. The term 'bursattee,' derived from the Indian words 'bururs' or 'bursat,' meaning rain or rain sore, was used in India to describe this condition. Moreover, equine pythiosis has been referred to as swamp cancer in some areas of the world. Thus, a relationship between wet habitats and this disease had been suspected from early in its history. In Australia, equine pythiosis is usually observed in late summer and autumn after the formation of large bodies of stagnant water from the remnants of the previous winter's rains and high seasonal temperatures – conditions that favor the rapid growth of *P. insidiosum* (Miller and Campbell 1982b; Shipton 1985). Miller (1983) noted that, under laboratory conditions, the zoospores of *P. insidiosum* are attracted to animal hair and tissue. He used horse hair as bait to isolate *P. insidiosum* from swampy areas in Australia. He speculated that this oomycete lives in wet habitats and that perhaps it may require a plant to complete its life cycle. Although an unidentified water lily was suggested as a possible plant host (Miller 1983),

other findings (Mendoza and Prendas 1988; Chaiprasert et al. 1990; Mendoza et al. 1993) indicate that, during its life cycle, *P. insidiosum* may preferentially use grass tissue, but in environments free of gramineae, it may complete its life cycle on other plants.

H135.4 ## DISTRIBUTION

P135.13 Pythiosis is a disease of the temperate, subtropical, and tropical areas of the world. It has been reported in Argentina, Australia, Brazil, Colombia, Costa Rica, Haiti, India, Indonesia, Japan, New Guinea, New Zealand, South Korea, Thailand, the USA, and Venezuela. Verified cases of this disease have not been reported in Europe. A case of cutaneous granulomas in a horse with all the features of pythiosis was, however, published by Drouin (1896) in France. The geographical location and tropical climate of Africa seemingly would make it an ideal region for pythiosis. Nevertheless, cases from that continent have yet to be reported. In the Americas, the disease has been diagnosed in North, Central, and South America, as well as the Caribbean islands.

P135.14   In the USA, the disease is more common in states near the Gulf of Mexico: Alabama, Florida, Louisiana, Mississippi, and Texas. In addition, sporadic cases have been diagnosed in nearby states such as Georgia, Missouri, North Carolina, South Carolina, Tennessee, and as far off as Illinois, Indiana, New York, and Wisconsin – all temperate regions of the USA near the Canadian border. Cases of pythiosis have not as yet been described from Mexico. In Central America, Costa Rica is the country with the largest number of documented cases of pythiosis in horses. This disease is most prevalent in the Atlantic region. Some cases have been diagnosed along its Pacific coast, however. The disease has been observed in other Central American countries – Guatemala, Nicaragua, and Panama (personal communications), but there are no recorded cases in the literature. In the Caribbean region, a case of human pythiosis was recently recorded in Haiti, so the disease may also be present in the other islands of that tropical region (Virgile et al. 1993). Northern Argentina, Brazil, Colombia, and Venezuela have reported cases of equine pythiosis since 1965. The Pantanal region of Brazil, the world's largest freshwater wetland, may have the highest incidence and prevalence of equine pythiosis, but systematic studies are not available to support this impression (dos Santos and Londero 1974; Santurio et al. 1998; Leal et al. 2001).

P135.15   Australia, the Pacific islands, and Asia have reported cases of pythiosis since the last century. In Australia, it has been found in Maitland, New South Wales, the coast of Queensland, the Northern Territory, and Western Australia. In Indonesia, the disease occurs in Borneo, Java, and Sumatra. In Japan, pythiosis is known to occur along the southern coast of Kyushu and the Ryukyu Islands. A case of intestinal pythiosis was recently reported in a dog from South Korea, suggesting that the disease could also be present in North Korea and China (Sohn et al. 1996). In Thailand, cases of human pythiosis have been found in the northern and southeastern parts of the country. Figure 24.5 shows the currently known distribution of pythiosis around the world.

H135.5 ## LIFE CYCLE OF *PYTHIUM INSIDIOSUM*

P135.16 *P. insidiosum*, as with other *Pythium* spp., is an organism of aquatic environments, although it can also be found in soil as a result of its ability to produce resistant spores (de Cock et al. 1987; Mendoza et al. 1993). To maintain its life cycle in nature, *P. insidiosum*

FIG135.5   **Figure 24.5** *Map showing the distribution of pythiosis in the tropical, subtropical, and temperate regions of the world.*

requires a low concentration of ions, a pH near neutrality, and a plant host (Shipton et al. 1982; Shipton 1983, 1985). As for other zoosporic fungal-like organisms (Endo and Colt 1974), *P. insidiosum* probably uses plants to produce sporangia and release zoospores to colonize other plants and expand its ecological niche, but it has not been demonstrated to cause pathology in plants. Early studies on the life cycle of this oomycete have shown that *P. insidiosum* is present in stagnant waters and may possibly require the Australian water lily or other plants to complete its life cycle (Miller 1983). It was also found that its zoospores may play an important role in the propagation of infections among plants and animals. More recently, other investigators (Mendoza et al. 1993) confirmed that *P. insidiosum* has a special tropism for animal and plant tissues, and that, upon release of zoospores, several mechanisms are activated that permit plant and animal tissue invasion. Encysted zoospores are surrounded by an amorphous material that has adhesive properties. This substance attaches the zoospores to the host's surface. They further hypothesized that chemotactic factors may signal zoospores to produce this material. Figure 24.3 illustrates various stages of *P. insidiosum*'s life cycle in detail.

H135.6
CLINICAL AND PATHOLOGICAL SIGNS OF INFECTIONS CAUSED BY *PYTHIUM INSIDIOSUM*

P135.17   The pathogenesis of pythiosis is not clear, in part because the disease is not reproducible in natural hosts. Laboratory data obtained from strains of *P. insidiosum* indicated that zoospores have a tropism for animal and plant tissues (Miller 1983; Mendoza et al. 1993). Thus, the zoospores, as motile propagules, in all likelihood, play a role as infecting agents in natural infections. However, reports of pythiosis in animals and humans with no history of contact with stagnant water open the possibility of inoculation by other propagules of *P. insidiosum* (hyphae, resting spores, oogonia) (Rinaldi et al. 1989; Fischer et al. 1994).

P135.18   When an animal or human enters swampy areas inhabited by *P. insidiosum*, its zoospores would be attracted by any open wound on their skin and encyst on the exposed tissue. On the basis of electron microscopic observations (Mendoza et al. 1993), the zoospores are known to secrete a sticky substance that allows them to make and maintain tight contact with the host during the initial stages of invasion. Stimulated by the temperature of the host's skin, the encysted zoospores develop a germ tube, often directed towards injured tissue (Mendoza et al. 1993), and mechanically penetrate the tissue (see Figure 24.2e). Ravishankar and Davis (2001) experimentally established that *P. insidiosum* cannot penetrate normal skin, but has the potential to successfully penetrate injured skin. Their study strongly

supports previous speculations. *P. insidiosum* initiates a cell-mediated immune response in its host, mostly in the form of eosinophils and a few neutrophils (Miller and Campbell 1983; Mendoza and Alfaro 1986), but the immune response cannot prevent the propagation of *P. insidiosum*. The eosinophils then, in an effort to phagocytose the microorganism, degranulate over the hyphae. New eosinophils fuse around the degranulated eosinophils and are also degranulated in turn. A mass known as a 'kunker' is thus formed. The eosinophilic material (Splendore–Hoeppli-like phenomenon) around *P. insidiosum* hyphae is a main feature of the infection in equines but not in the other species. Kunkers are composed of degranulated eosinophils interlaced with the viable hyphae of *P. insidiosum*. The lower panel of Figure 24.3 summarizes the probable mechanism of infection. The tissue damage observed in acute and chronic cases has been attributed to the release of chemicals from the degranulated eosinophils and mast cells (Miller 1981; Miller and Campbell 1983; Mendoza et al. 2003).

Pythiosis in non-primates

HORSES

Since the beginning of this century, the clinical features   P135.20 of pythiosis in horses have been well described by several authors (de Haan and Hoogkamer 1901, 1903; Witkamp 1924; Bridges and Emmons 1961; McMullan et al. 1977; Miller and Campbell 1982b; Chaffin et al. 1995). There are no reports of predisposition with regard to sex or age of the afflicted animals. Lesions caused by *P. insidiosum* are found on any part of a horse's body, although they are more common on the lower limbs because these are the first areas to come in contact with swampy water. In addition, the lesions in horses can also be found on the thorax, abdomen, neck, shoulders, genitalia, and head. Pythiosis often occurs as a single lesion but unusual cases with multicentric granulomas have been encountered. When lesions develop on the extremities (particularly near the joints), lameness is a frequent finding. In chronic pythiosis, emaciation and secondary infections are also common. There are no reports of animal-to-animal, or animal-to-human transmission of infection.

Lesions are circular in shape, from 5 to 500 mm in   P135.21 diameter (Miller and Campbell 1982b; Mendoza and Alfaro 1986), with a characteristic serosanguineous discharge (Figure 24.6a, b). Intense pruritus is one of the characteristic clinical features of the disease in horses. Ulceration of the tissue is common in large lesions. Smaller ones often show one or more small ulcers. As the disease progresses, small coral-like masses called kunkers, which contain viable hyphae of *P. insidiosum*, are found in the sinuses and surface of the infected tissue. They vary in size and form (10–90 mm in

Figure 24.6 *Clinical features of cutaneous granulomas caused by* Pythium insidiosum *in two horses (a, shoulder; b, leg). Note the circular shape typical of pythiosis granulomas in horses.*

diameter) (Figure 24.7). Metastasis of *P. insidiosum* through lymphatic vessels to regional lymph nodes (Connole 1973; Murray et al. 1978), lungs (Witkamp 1924; McMullan et al. 1977; Goad 1984), or bones (Mendoza et al. 1988; Alfaro and Mendoza 1990; Eaton 1993; Neuwirth 1993) is common (Figure 24.8). Equine intestinal pythiosis, resulting from direct inoculation of propagules of *P. insidiosum* in mucous membranes, has been reported (Brown and Roberts 1988; Morton et al. 1991; Purcell et al. 1994).

Histopathologically, in the early stages of pythiosis, abundant microabscesses with an inflammatory reaction, composed mainly of eosinophils, a few neutrophils, lymphocytes, and macrophages, are present in the subcutaneous lesions. In chronic cases, eosinophilic granulomatous tissue with giant cells is often recorded. In central areas of inflammation, sequestered eosinophilic masses (kunkers) are detectable. Sections of the kunkers stained with Gomori's methenamine silver reveal the presence of aseptate hyphae 6–10 μm in diameter along with cellular debris (Figure 24.9).
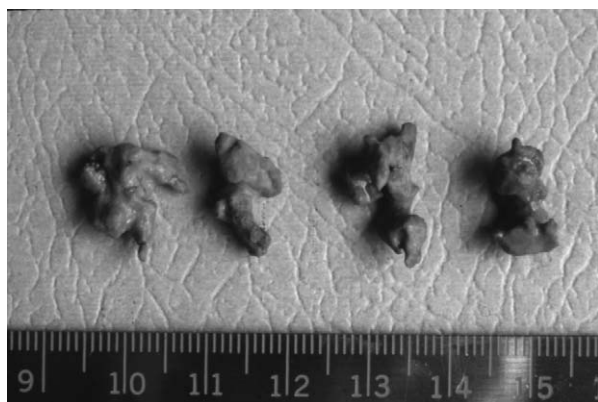

Figure 24.8 *Posterior view of a bone in the front leg of a horse affected by* Pythium insidiosum, *with lysis and exostosis of the metacarpal, carpal, and distal radius, and the first phalanx bones.*

DOGS

Canine infections were first reported in dogs with cutaneous and gastrointestinal lesions from the Gulf of Mexico in the USA (Heller et al. 1971; Miller et al. 1983; Pavletic et al. 1983; Foil et al. 1984). Subcutaneous pythiosis lesions have been recorded also on the legs, face, and tail in dogs (Figure 24.10) (Foil et al. 1984;

Figure 24.7 *This shows four irregularly shaped, firm, coral-like masses called kunkers which were removed from a cutaneous granulomatous lesion in a horse with pythiosis. (Reproduced, with permission, from Mendoza and Alfaro 1986)*
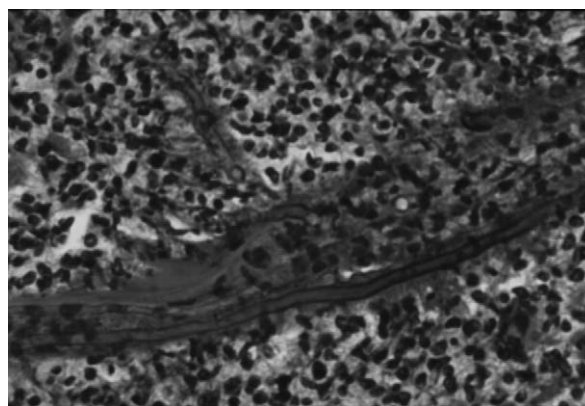

Figure 24.9 *Photomicrograph of a kunker's tissue section, showing the aseptate hyphae of* Pythium insidiosum *and cellular debris. Periodic acid–Schiff stain; magnification × 9 200.*
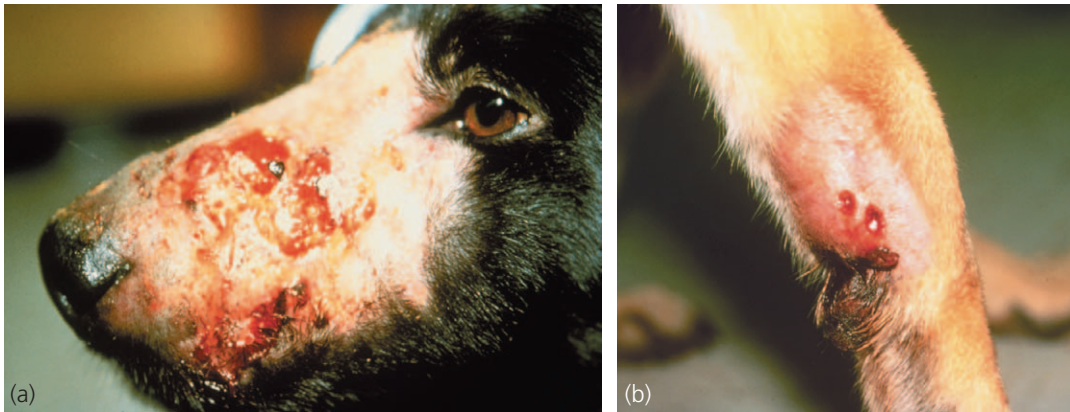
Figure 24.10 *(a, b) Skin lesions caused by* Pythium insidiosum *on two dogs with subcutaneous infection. (Courtesy of Dr Randall C. Thomas)*

Thomas and Lewis 1998). Systemic pythiosis with involvement of internal organs has also been reported in dogs (Foil et al. 1984; Thomas and Lewis 1998). The pruritic skin lesions were denuded of hair and perforated by fistulous sinus tracts discharging a serosanguineous exudate. Histopathological findings included multifocal areas of necrosis with moderate neutrophils and macrophages. Discrete granulomatous infiltrates with eosinophilic material are surrounded by eosinophils, neutrophils, and epithelioid cells. Giant cells have also been reported in chronic cases. Hyphae which measured 4.4–8.6 μm in diameter were detected in these tissues.

P135.24   Gastrointestinal pythiosis in dogs is characterized by vomiting, weight loss, and sporadic diarrhea (Miller et al. 1983, 1985; Pavletic et al. 1983; Thomas and Lewis 1998). Formation of hard gastrointestinal granulomatous masses, areas of mural thickness, and mucosal ulceration are common in most cases. Lesions can spread to adjacent tissue such as that of the pancreas, uterus, and mesenteric lymph nodes. Histopathologically, the mucosa shows ulceration, atrophy, and epithelial cell hyperplasia. The submucosa is thickened and the muscular mucosa contains focal granulomas. Neutrophils, eosinophils, plasma cells, macrophages, epithelioid cells, and giant cells are observed in the affected tissues. The hyphae of *P. insidiosum* are difficult to detect in hematoxylin–eosin-stained sections, but with Gomori's methenamine silver stain, hyphae between 2.5 and 8.9 μm in diameter are readily detected. Recently, cases of canine pythiosis have been recorded in Australia (English and Frost 1984) and in the USA (Miller et al. 1985).

P135.25   Grooters (2003) stated that there were clinical cases similar to those observed in canine pythiosis, caused by an undescribed species in the genus *Lagenidium*. She based her findings on histopathological, cultural, and molecular analysis. However, her data have yet to be confirmed by others (see earlier under Taxonomy and morphological features of *Pythium insidiosum* H135.2).

Cats and cattle

P135.26   Few cases of pythiosis in cats (Bissonnette et al. 1991; Thomas and Lewis 1998) and calves (Miller et al. 1985; Santurio et al. 1998) have been described in the literature. Bissonnette et al. (1991) was the first to diagnose a case of pythiosis in a cat with facial swelling, but none of its internal organs was involved (Figure 24.11a). *P. insidiosum* was isolated from the affected tissue. Histopathological features, similar to those recorded in horses and dogs, were observed in this case.

P135.27   A total of five calves with pythiosis have so far been reported with the disease. Showing multiple focal ulcers and fistulous tracts, draining a watery purulent exudate and with swelling of fetlock joints, they were diagnosed with pythiosis (Miller et al. 1985; Santurio et al. 1998). Miller and colleagues (1985) reported that *P. insidiosum* was isolated from two of the studied calves, whereas *P. insidiosum*-like hyphae were found in the tissue of a third case. Histological sections from that case showed granular encrustations around the hyphae. The perihyphal deposit was composed of granular material similar to that of the Splendore–Hoeppli phenomenon. Similar observations were described by Santurio et al. (1998) in calves from the Pantanal region of Brazil.

P135.28   More recently, several cases of pythiosis in calves were also described in the State of Apure, Venezuela (Figure 24.11b) (personal communication, Rosa Cristina Perez). Serological testing done in these animals was consistent with *P. insidiosum*. In addition, histopathological and DNA sequencing analysis from the infected tissues confirmed the presence of this pathogen in the infected calves.

Human pythiosis

P135.29   Most human cases have occurred in Thailand with only a few reports of such cases in Australia, Haiti, New

(a)

(b)

FIG135.11 **Figure 24.11 (a)** *Lesions on a cat from Florida, USA with subcutaneous pythiosis (arrow).* **(b)** *A subcutaneous lesion on the limb of a Venezuelan calf caused by* Pythium insidiosum. *(Courtesy of Dr Randall C. Thomas and Rosa Cristina Perez)*

(a)

(b)

Figure 24.12 (a) *Dry gangrenous lesion on the left leg caused by* Pythium insidiosum *in a Thai thalassemic patient.* **(b)** *Digital subtraction angiography of the same patient showing occlusion of the left superficial femoral artery. (Courtesy of Dr Ploenchan Chetchotisalkd, Thailand)* FIG135.12

Zealand, and the USA. Two human cases of subcutaneous pythiosis were mentioned by de Cock et al. (1987), but details about those early cases were not available. The first five cases of this disease in humans involved two women and three men in the rural areas of northern Thailand (Sathapatayavongs et al. 1989). Ten more cases were later reported from the same areas of Thailand (Chetchotisakd et al. 1992; Wanachiwanawin et al. 1993). Most were associated with a hemoglobinopathic syndrome. Clinically, progressive gangrene and pain in the extremities were the main findings. Strikingly, all cases showed large vessel arteritis (Figure 24.12). Angiographic analysis indicated occlusion of the iliac, popliteal, and femoral arteries. Treatment by amputation was successful in seven of the 15 cases. Fatal invasion of the abdominal arteries with occlusion of the infrarenal part of the aorta was also recorded. So far, the majority of cases of human pythiosis have occurred in Thailand, with some 90 new patients reported since

the first case in 1987 (personal communication, Theerapong Krajaejun).

Three patients with subcutaneous orbital pythiosis were diagnosed in the USA (Rinaldi et al. 1989). One was a healthy boy in Texas who had accidentally been struck in his right eye. He developed progressive periorbital oedema with chemosis, erythema, and periorbital cellulitis (Figure 24.13). *P. insidiosum* was isolated from biopsied tissue. Details of the second case were not discussed. The third case was recorded by Shenep et al. (1998) on a 2-year-old boy from Tennessee. Two additional cases of pythiosis with a similar clinical history were reported in Australia (Triscott et al. 1993). These cases involved an 11-year-old and a 14-year-old boy. Both developed periorbital swellings which rapidly developed into an orbital tumour. The diagnosis was made histopathologically using an immunoperoxidase staining assay (see section on Immunohistochemical assays P135.45 below). In addition to vessel arteritis and subcutaneous pythiosis in humans, cases of keratitis have also been recorded in Haiti (Virgile et al. 1993), New Zealand (Fraco and Parr 1997), and Thailand (Kunavisarut et al. 1988; Imwidthaya and Methitrairut 1992; Imwidthaya 1995).

Triscott et al. (1993) described in detail the histopathology of subcutaneous pythiosis in humans. They found that the histological features were similar to those of equine pythiosis. Eosinophilic granular masses of about 7 mm in diameter, containing degenerating eosinophils, eosinophilic granules, hyphae, and intact eosinophils at the edge of the granulomatous areas were the main findings. They also reported a mixed inflammatory cell infiltrate comprising eosinophils, lymphocytes, neutrophils, macrophages, and mast cells. As in equine pythiosis, the hyphae of *P. insidiosum* were restricted to the areas of the eosinophilic granular masses. Kunkers, as recorded in equine pythiosis, have not, however, been detected in tissue from human cases.

FIG135.13 **Figure 24.13** *Severe orbital swelling resulting from* Pythium insidiosum *infection in a healthy boy. (Courtesy of Drs Michael G. Rinaldi and Steven Seidemseld, USA)*

H135.7 ## IMMUNOLOGY

P135.32 The development of an immune response to *P. insidiosum* antigens during equine infections has been known since early in the century (Witkamp 1924, 1925). In those studies, Witkamp described precipitin and complement fixation antibodies, and a cellular immunity response to a skin test in horses with active pythiosis. Miller and Campbell (1982a), using a similar approach, confirmed this early work. At least three antigens were prepared. A trypsin–hyphal antigen detected one precipitin band by double immunodiffusion (ID) in all horses with the disease. By contrast, only 82 percent of the horses were positive to a complement fixation test. The skin test, using a precipitate protein antigen, was positive in 64 percent of the clinically infected horses and in 31 percent of normal horses inhabiting the enzootic areas. The finding that normal animals reacted positively in a delayed hypersensitivity skin test indicates that subclinical pythiosis may occur in some horses. They also reported detectable levels of *P. insidiosum* antibodies at the day of birth in foals born to mares with active pythiosis, suggesting that their foals received some degree of passive immunity against *P. insidiosum*.

Horses with a history of pythiosis but negative to skin tests were considered anergic. Analogous immunological studies in other areas also confirmed these findings (Mendoza and Alfaro 1986; Grooters et al. 2002; Krajaejun et al. 2002).

P135.33 Immunization (immunotherapy) with antigens derived from *P. insidiosum* was shown to have curative properties in horses afflicted with the disease (Miller 1981; Mendoza and Alfaro 1986; Hensel et al. 2003; Mendoza et al. 2003). Fifty percent of infected horses responded to immunization. Changes from an eosinophilic infiltrate, before immunization, to a mononuclear response (macrophages, T lymphocytes), after immunization, suggested that cellular immunity was playing a major role in the clearance of *P. insidiosum* from immunized horses. Mendoza et al. (2003) suggested that a switching from a T helper 2 (Th2) to a T helper 1 (Th1) response was behind the curative properties of the immunotherapeutic antigens used by these investigators.

P135.34 Animals in the early stages of pythiosis react positively to a delayed hypersensitivity skin test using a culture filtrate antigen (CFA) and are cured by immunization with precipitated protein antigens. These observations, together with the fact that sera from humans and animals reacted positively in ID and Western blot tests, indicated that cellular and humoral immunity were active in the early stages of the disease. Animals became anergic when the disease reached chronicity (>2 months).

P135.58 Systemic human pythiosis in Thailand, on the other hand, has been diagnosed mainly in thalassemic patients (Imwidthaya 1994a). This may indicate that the disease usually occurs in debilitated patients. However, there is no such parallel in animals with this disease. Moreover, in the USA and Australia, four subcutaneous human cases of pythiosis were recorded in healthy individuals (Rinaldi et al. 1989; Triscott et al. 1993). Thus, the predisposition to *P. insidiosum* infection in patients with thalassemia hemoglobinopathy syndrome is intriguing and merits further investigation.

LABORATORY DIAGNOSIS   H135.8

P135.35 Clinical material from suspected cases of pythiosis should be sent directly to the laboratory for processing. Storage of tissue samples at 4°C during transportation may result in the death of *P. insidiosum*. If the samples cannot be delivered immediately, it is recommended that they should be vigorously washed several times in sterile distilled water, and then transported in sterile distilled water plus antibiotics (ampicillin + streptomycin). The most suitable tissue from which to isolate *P. insidiosum* from horses is the coral-like structures known as kunkers. These masses are removed and repeatedly washed in distilled water. The tissue or kunkers are then cut into small pieces, placed on Sabouraud's dextrose agar (SDA) and incubated at 37°C for 24–48 hours. Parts of the pieces of the kunkers should be placed in 10

percent KOH for direct microscopy examination. Wet mounts are of value for the early detection of *P. insidiosum*, which appear as hyaline sparsely septated hyhae 4.0–9.0 μm in diameter.

P135.36   Several media have been used for the isolation of *P. insidiosum*. The most common is SDA. *P. insidiosum* grows rapidly at 37°C on that medium. In 24 hours it develops flat colonies that are 20 mm in diameter. Microscopically, however, the hyphae are found to be sterile at this stage. The hyphae are branched at approximately 90° angles and are usually coenocytic, although occasional septa are observed in tissue sections and in old cultures. To induce zoospore production, it is recommended that boiled grass leaves be placed on *P. insidiosum* cultures. After 24 hours of incubation at 37°C, the leaves are immersed in dilute salt solution containing calcium and incubated 2–3 hours at 37°C (Mendoza and Prendas 1988). Zoosporangia containing motile zoospores will be readily observed at the edges of the leaves. Oogonium production is very rare in *P. insidiosum*, so specific identification is based on serological tests and DNA testing (Mendoza et al. 1987; Badenoch et al. 2001; Grooters and Gee 2002; Schurko et al. 2003a).

Histopathology

P135.37   In tissue sections, the hyphae of *P. insidiosum* in tissue sections are often difficult to differentiate from those found in cases of zygomycosis caused by fungal species of the orders Mucorales and Entomophthorales. In such cases, the diameter of the hyphae may be of help. The hyphae of *P. insidiosum* are between 3.0 and 10.0 μm in diameter, whereas those of *Basidiobolus ranarum* and *Conidiobolus coronatus* are broader, being about 5–15 μm in diameter. However, the isolation of *P. insidiosum* and the use of serological tests are the ultimate basis for the diagnosis of pythiosis. In some cases, clinical data can also be useful. For example, clinically pythiosis and zygomycosis caused by *B. ranarum* occur in the same anatomic areas in horses, so it is often difficult to differentiate between them, although infections caused by *C. coronatus* occur primarily in a horse's nostrils. This clinical difference may be of help, if serological tests and isolation of the etiologic agent are not possible.

DNA-based diagnostic assays

P135.38   The identification of *P. insidiosum* in the laboratory and histopathological preparations is entirely based on its sexual oogonia and hyphae-like morphological features. However, several fungi have also morphological similarities with the filamentous structures of this oomycete, and the development of oogonia in agar plates is difficult. To overcome these facts, DNA methodologies have recently been used for its diagnosis and identification (Badenoch et al. 2001; Grooters and Gee 2002; Reis et al. 2003). Badenoch et al. (2001) were the first to use DNA sequencing to identify *P. insidiosum* hyphae from a patient with keratitis. These authors suggested that molecular tools could be important in differentiating *P. insidiosum* from the filamentous fungi. Later, Grooters and Gee (2002) developed nested primers that specifically amplified 105 base pairs from *P. insidiosum*'s genomic DNA. More recently, Reis et al. (2003) obtained several amplicons of *P. insidiosum*'s 18S small-subunit rDNA sequences from three cases of horses with systemic pythiosis. These studies indicated that DNA-based technology is ideal to identify *P. insidiosum* from fixed tissues and cultures, and could be successfully used more frequently in the future.

Serology

Serological methods for diagnosing *P. insidiosum* infections were developed in the beginning of the century (Witkamp 1925). Miller and Campbell (1982a) and Mendoza and Alfaro (1986) based their immunological studies of equine pythiosis on Witkamp's early work. They found that immunodiffusion and complement fixation tests were useful in the diagnosis of pythiosis. Moreover, a skin test was shown to be effective in determining subclinical infections in animals with active disease.   P135.39

IMMUNODIFFUSION TEST

Two antigens have been used to diagnose *P. insidiosum* infections by immunodiffusion. One was prepared from the hyphae of *P. insidiosum* digested with trypsin (Miller and Campbell 1982a). This trypsin antigen detected one precipitinogen in all animals with active pythiosis. However, its shelf life was short. The other antigen was obtained from concentrated CFAs (Mendoza et al. 1986). It detected three to six precipitins in sera from horses and humans with pythiosis. One of them was the precipitinogen reported earlier in an ID test with trypsin antigens. Healthy individuals were always negative with the ID test. Chronic equine cases of pythiosis showed no bands in the ID test. These animals also did not react to skin tests and were considered anergic. The ID test detects pythiosis in its early stages (3 days), indicating that antibodies against the antigens of *P. insidiosum* are developed early in the course of infection.   P135.40

No crossreactivity was recorded using the CFA of *P. insidiosum* and sera from patients with zygomycosis or other mycotic and bacterial diseases. The ID test was specific for pythiosis. Thus, the presence of a precipitin band in ID is suggestive of this disease. The number of precipitins in ID does not correlate with the severity of the disease. Horses treated and recovered from the disease showed no precipitin bands after 2 months of   P135.41

successful treatment. Some bands of non-identity were recorded when sera from horses with pythiosis reacted to the antigens of *B. ranarum (haptosporus)* or *C. coronatus* (Kaufman et al. 1990). The CFA proved to be more sensitive, stable and useful, not only in diagnosing the disease in cats, cattle, dogs, horses, and humans, but also in monitoring response to treatment (Mendoza et al. 1986; Imwidthaya and Srimuang 1989; Pracharktam et al. 1991). In addition, complement fixation has been previously used (Miller and Campbell 1982a). However, this test is no longer available.

WESTERN BLOT

P135.42   Culture-filtrated antigens in the ID test detected at least six precipitinogens. However, ID is too insensitive and also unable to identify major antigenic immunogens. Western blot analysis was introduced to determine which of the antigens of *P. insidiosum* were of importance during infection (Mendoza et al. 1992a). It was found that equine IgG recognized almost all of the cytoplasmic proteins of *P. insidiosum*. Several protein antigens of 28, 30, and 32 kDa) and other immunogens were found to be immunodominant (Figure 24.14). Negative results in Western blot were recorded using sera from healthy horses or sera from horses with various other diseases. Equine immunoglobulin G against the 32, 30, and 28 kDa and other immunodominant antigens was found to persist at least for a year in horses cured by immunotherapy. This suggests that these prominent antigens may also be important as protective immunogens in horses. It was later found that the addition of cytoplasmic antigens, containing the 28, 30, and 32 kDa immunodominant proteins, to the available *Pythium*

FIG135.14 **Figure 24.14** *Immunoblot analysis of the CFAs from different strains of* Pythium insidiosum *(lanes 1–5) and* Conidiobolus coronatus *CFAs (lane 6) after reacting with sera from horses with pythiosis. The 28, 30, and 32 kDa proteins are observed as prominent bands. A band of crossreaction was observed against* C. coronatus's *CFA (lane 6). (Reproduced, with permission, from Mendoza et al. 1992a)*

vaccine enhanced its curative properties (Mendoza et al. 2003). This finding suggested that these proteins may play a major role in the immunotherapy of pythiosis. In equine chronic pythiosis, Western blot fails to detect the cytoplasmic antigens of *P. insidiosum*, indicating that anti-*P. insidiosum* IgG is absent from their sera. This supports the concept that horses with the chronic disease are anergic. IgG from horses with active pythiosis recognized only the 44 kDa cytoplasmic antigen of *C. coronatus*, confirming that the Western blot test is very specific in horses. This test is useful for analyzing immunoglobulin classes during infection. Recently, a single-step immunoblot (SIB) assay to diagnose pythiosis in horses was shown to be sensitive, specific, and easy to perform (Rosa 1993). Studies using human sera in Western blot are not yet available.

ENZYME-LINKED IMMUNOSORBENT ASSAY

The enzyme-linked immunosorbent assay (ELISA) test   P135.43
was originally designed to detect anti-*P. insidiosum* IgG in proved cases of pythiosis with a negative ID (Rosa 1993; Mendoza et al. 1997). More recently, the test was shown to be helpful in cases of a dog and cat with the disease (Mendoza et al. 1997; Grooters et al. 2002). In Thailand, at least another in-house assay was developed using antigens from Thai isolates (Krajaejun et al. 2002). These ELISAs showed high sensitivity and specificity to diagnose pythiosis and were also suitable to monitor the response to treatment, confirming previous observations (Mendoza et al. 1997). In addition, some healthy horses from enzootic regions reacted positively in low titers, suggesting that subclinical infection may occur.

The ELISA developed to detect pythiosis in humans   P135.44
(Krajaejun et al. 2002) and animals (Mendoza et al. 1997; Grooters et al. 2002) both showed 100 percent sensitivity and specificity. These ELISAs were capable of discriminating between sera from apparently healthy individuals or heterologous infections and patients with active pythiosis.
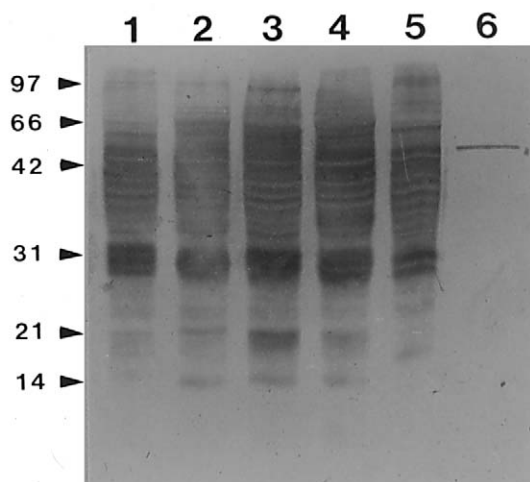
IMMUNOHISTOCHEMICAL ASSAYS

Two tests have been developed for the immunodetection   P135.45
of *P. insidiosum* hyphae in tissue: an immunofluorescence test (Mendoza et al. 1987) and an immunoperoxidase assay (Brown and Roberts 1988; Triscott et al. 1993). The immunofluorescence assay was shown to be entirely specific for *P. insidiosum* in the detection of its hyphae in cat, dog, and human tissues. However, in equine pythiosis, tissue sections of kunkers containing hyphae showed a uniform fluorescence throughout the sections. This was attributed to the exoantigens released by the hyphae of *P. insidiosum* into the matrix of the kunkers. Immunofluorescence has also been used to differentiate *P. insidiosum* from other species of *Pythium*.

The immunoperoxidase assay was performed on the tissue of dogs, horses, and humans with the disease (Brown and Roberts 1988; Triscott et al. 1993; Fischer et al. 1994). *P. insidiosum* in those tissues stained positively after applying anti-IgG peroxidase. Tissue sections from cases of zygomycosis caused by *B. ranarum* and *C. coronatus* were negative in these assays. Both assays were found to be sensitive and specific.

ANIMAL INOCULATION

All attempts experimentally to reproduce pythiosis in dogs, horses, and mice have failed (Patino-Meza 1988). Early workers, however, found that rabbits were susceptible to infection by the propagules of *P. insidiosum* (Witkamp 1924). Amemiya (1969, 1982), working with strains isolated from horses with granular dermatitis in Japan, confirmed the susceptibility of rabbits to hyphal inoculation. In his work, he found that subcutaneous inoculation of *P. insidiosum* hyphae gave rise to nodules that contained hyphae and inflammatory cells at the injection sites (Figure 24.15). Intravenous injection caused systemic infections in the inoculated rabbits with granulomatous necrotizing masses in the aortas, intestines, livers, and lungs.

Miller and Campbell (1983) evaluated motile zoospores as inocula in cortisone-treated and non-cortisone-treated rabbits. They found that both groups were extremely susceptible to infection by the zoospores of *P. insidiosum*. All inoculated animals developed necrotizing hepatitis or embolic nephritis, thus demonstrating that *P. insidiosum* does not require a debilitated or immunocompromised host to induce infections. In striking contrast, numerous authors have failed to reproduce the disease in horses and dogs, even after exposing them to motile zoospores by submerging the animals in tanks of seeded water which simulated the conditions encountered in swamps. The predisposing factors required to develop pythiosis in dogs, horses, and humans remain unknown. Mendoza

Figure 24.15 *Experimental pythiosis in a rabbit. Subcutaneous nodules and systemic dissemination of* Pythium insidiosum *are the main features of such experimental infections.*

et al. (2003), based on clinical data, however, argued that humans and animals are resistant to pythiosis. They also suggested that perhaps a defect in the immune response is what makes a host susceptible, which in part explains the low occurrence of the infection in the endemic areas.

TREATMENT

Treatment of infections caused by *P. insidiosum* in animals and humans is difficult. Three therapeutic methods are, however, often used for this disease: surgery, drugs, and immunotherapy.

Surgery

Radical surgery has been successfully used in cases of equine pythiosis since the last century. It consists of removal of the lesions and their kunkers, followed, in some cases, by cauterization (Habbinga 1967; McMullan et al. 1977). This method is very popular and frequently used by veterinary practitioners, although it is not always successful. In general, the response to surgery is limited. Moreover, lesions on the limbs of equines are not easily treated by this method because of their delicate anatomic structure. Surgical removal of tumor-like lesions in dogs with intestinal pythiosis has been reported, but survival beyond 3 months was extremely rare in the treated dogs (Fischer et al. 1994; Thomas and Lewis 1998). A common shortcoming of surgical treatment is recurrence as a result of incomplete removal of infected tissue.

Radical surgery has also been used in human cases with arteritis caused by *P. insidiosum*. Amputation of the extremities is a drastic procedure of the last resort, which is used to treat patients with severe *Pythium* arteritis. However, this method has proved to be only partially successful because most patients finally died of disseminated abdominal arteritis (Sathapatayavongs et al. 1989; Wanachiwanawin et al. 1993). Early detection of affected vessels using angiography is important in determining the most appropriate amputation site.

Chemotherapy

Two main groups of drugs are commonly used to treat pythiosis: iodide and amphotericin B. Iodides have been used since early in the century in equine pythiosis with contradictory results. Although some authors reported cures with intravenous injections of potassium iodide (0.75 g/45 kg in weight) (Gonzalez et al. 1979) or sodium iodide (1.0 g/15 kg in weight) (Hutchins and Johnston 1972), other investigators reported failures (de Haan and Hoogkamer 1903; Hartsfield 1971; Murray et al. 1978). Similar observations were recorded in humans. Patients with arteritis caused by *P. insidiosum* did not respond to potassium iodide, but patients with subcutaneous

pythiosis seem to have responded well to this drug (Thianprasit 1990). The main drawback of iodide and amphotericin B is their toxicity (Murray et al. 1978).

P135.53   Amphotericin B is the drug of choice in many mycotic infections. As a result of the lack of ergosterols in the cytoplasmic membrane of members of the genus *Pythium*, however, one could predict that amphotericin B would be ineffective in infections caused by *P. insidiosum*. Nevertheless, in equine pythiosis, amphotericin B has been used with some success. Eight cases of equine pythiosis were cured out of a total of 10 horses (McMullan et al. 1977) after intravenous injection of amphotericin B (0.38–1.47 mg/kg). Topical application of this drug had little or no effect on equine cases (Miller et al. 1983). The use of amphotericin B has been limited partly as a result of the cost of therapy, the poor rate of success, and its toxic side effects. Amphotericin B in humans with pythiosis gave contradictory results as well. For example, intravenous injections of amphotericin B (0.1 mg/kg) were ineffective in cases with arteritis (Wanachiwanawin et al. 1993) and in one case of subcutaneous infection (620 mg/kg, over a 6-week period) (Rinaldi et al. 1989). It was effective in two subcutaneous pythiosis cases when it was used in combination with 5-fluorocytosine (amphotericin B 0.5 mg/kg/day, 5-fluorocytosine 150 mg/kg/day) (Triscott et al. 1993). A combination of itraconazole and terbinafine saved the life of a boy in Tennessee with subcutaneous pythiosis (Shenep et al. 1998). However, this combination has been also used in dogs and other animals with contradictory results.

Immunotherapy

P135.54   Immunization of equines with products derived from *P. insidiosum* cultures was reported to have curative properties in Australia (Miller 1981) and Costa Rica (Mendoza and Alfaro 1986). Two antigens, referred to in the literature as Miller's and Mendoza's vaccines (Newton and Ross 1993), have been used for immunization. The Australian vaccine was prepared from sonicated hyphal antigens, whereas the Costa Rican vaccine used precipitated proteins from CFAs. Immunotherapy of horses with pythiosis using Miller's vaccine alone gave a success rate of 53 percent. An increase in the percentage of cured cases was obtained when immunization was followed by surgical removal of cutaneous lesions. Similar results were obtained using Mendoza's vaccine (Figure 24.16). Nevertheless, significant reduction in the swelling, caused by the vaccine at the site of injection, an undesirable side effect of Miller's vaccine, was the main feature of Mendoza's vaccine. Moreover, the vaccine described by Miller was unstable, losing its curative properties after storage at 4°C. By contrast, Mendoza's vaccine was found to be effective even 18 months after its preparation.

Figure 24.16 (a) *A horse with pythiosis before immunotherapy;* **(b)** *the same horse after successful immunotherapy.* *(Reproduced, with permission, from Mendoza and Alfaro 1986)*

FIG135.16

P135.55   Recently Mendoza et al. (2003) introduced new immunotherapeutic immunogens to treat pythiosis in both humans and animals. This new formulation contains exo- and endoproteins extracted from cultures of *P. insidiosum* and was able to cure chronic and acute cases of the disease (Thitithanyanont et al. 1998; Hensel et al. 2003; Mendoza et al. 2003). These studies suggest that hyphal antigens may contain products that are involved in the enhancement of the immunological response to immunization. Horses with the disease that failed to respond to immunization did not develop swellings at the injection sites. All in all, this finding suggests that the response to immunization is directly related not only to the immunostatus of the infected hosts , but to the type of immunogens used.

P135.56   Based on histopathological data, the hyphae of this oomycete are always sequestered within kunkers. Thus, it has been postulated that perhaps some antigens are downregulated during infection (Mendoza et al. 2003). Reports on the histological changes that take place after immunotherapy had shown that the initial eosinophilic inflammatory reaction, typical of equine pythiosis, changed to a mononuclear response composed mainly of macrophages and T lymphocytes (perhaps cytotoxic lymphocytes) (Miller 1983; Mendoza and Alfaro 1986). The antigens, used during immunotherapy, apparently trigger a Th1 immune response with macrophages and cytotoxic lymphocytes that eventually clear the organism from tissues. This contention is supported by the fact that cultures of biopsied tissue in immunized horses are always negative for hyphae. This confirms, in part, the observation that the digestion of the kunkers by the mononuclear inflammatory cells seems to destroy the hyphae of *P. insidiosum* (Miller 1981; Mendoza et al. 1992b). Complications associated with immunotherapy are mainly an inflammatory reaction at the injection site, secondary bacterial contamination, and lameness in lesions located on limbs. It was reported that some horses cured by immunization relapsed one year later.
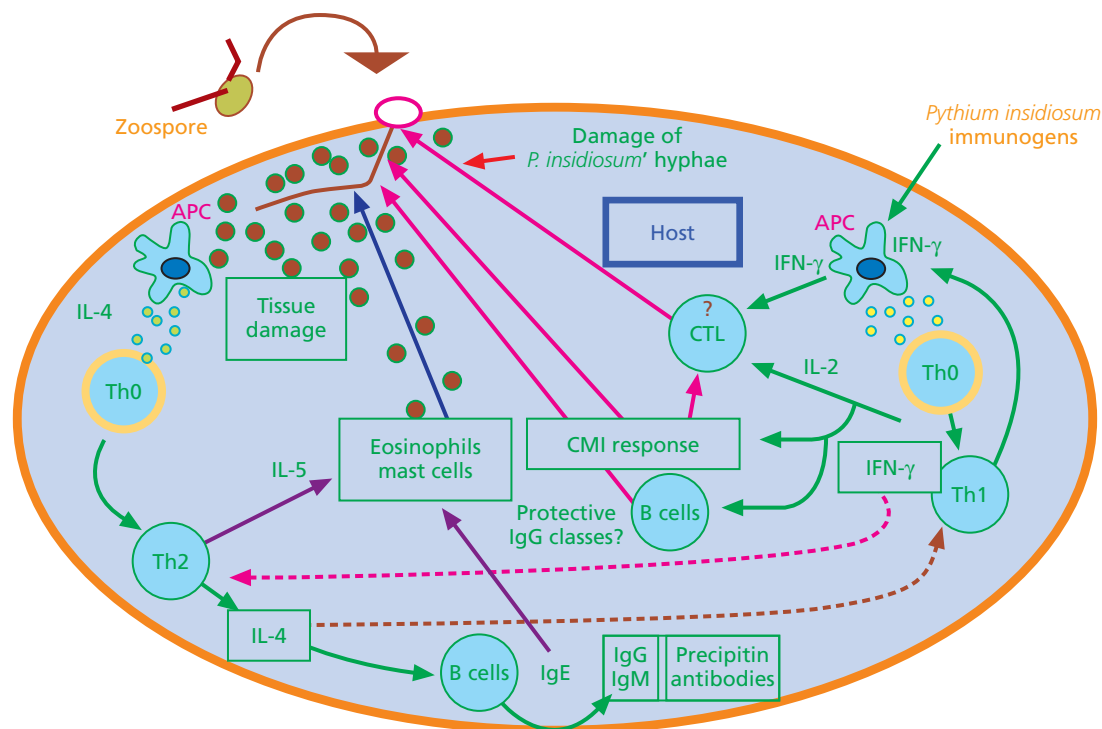
Figure 24.17 *Proposed working hypothesis of the pathogenic mechanisms involved during natural infection and those triggered by immunotherapy. In this model, pythiosis begins when the host contacted a zoospore (or other propagules of the pathogen) through a wound.* Pythium insidiosum *will develop hyphae-like filaments that penetrate the host. Once in the tissues, it will release antigens that will lock the immune system into a Th2 response and which contain typical cellular and other mediators including IL-4 that could downregulate the Th1 response (brown dotted arrow) and cause pythiosi (blue arrow) (left side of the figure). By contrast, after immunotherapy, IFN-γ is immediately triggered, which in turn activate a Th1 immunity and downregulates the Th2 response (pink dotted arrow). The mononuclear response (putative natural killers and cytotoxic lymphocytes, cell-mediated immunity) is believed to be responsible for the killing of the pathogen in the infected tissues (red arrows). APC, antigen presenting cell. (Reproduced, with permission, from Mendoza et al. 2003)*

This suggests that, if the vaccine has prophylactic properties, it is of short duration. The prophylactic and curative properties of *P. insidiosum* immunogens have been recently evaluated by Santurio and Leal (2003) in an animal model. These investigators confirmed that the antigens derived from this oomycete have, indeed, curative and prophylactic properties.

The mechanism involved in the response of horses to natural infection and to immunotherapy using *P. insidiosum* products has been recently addressed by Mendoza et al. (2003). In their model, *P. insidiosum*, after contact with the host, will release exoantigens that will lock the immune system into a Th2 response (eosinophils, mast cells, IgE, IL-4, IL-5, precipitin, IgG, and IgM). They propose that the degranulation of the eosinophils and mast cells is responsible for the tissue damage observed during natural infection (Figure 24.17). By contrast, after injection with the immunotherapeutic antigens of *P. insidiosum*, the host will trigger a Th1 response with interferon-gamma (IFN-γ) that eventually downregulates the Th2 response, and a prominent mononuclear response. These investigators speculated that the events triggered by the immunotherapeutic antigens ultimately kill the pathogen in the infected tissues,

and therefore are more likely responsible for the cure of the infected hosts (Figure 24.17).

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