New Insights into the *In Vitro* Susceptibility of *Pythium insidiosum*

Érico S. Loreto,^a Juliana S. M. Tondolo,^a Maiara B. Pilotto,^a Sydney H. Alves,^b Janio M. Santurio^a

Programa de Pós-Graduação em Farmacologia, Centro de Ciências da Saúde, Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brazil^a; Programa de Pós-Graduação em Ciências Farmacêuticas, Centro de Ciências da Saúde, Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brazil^b

We have determined the *in vitro* activity of several antibacterial and antifungal drugs against *Pythium insidiosum* using broth microdilution (BMD), disk diffusion, and Etest methods. The largest zones of inhibition (disk diffusion) and the lowest BMD and Etest MICs were observed for azithromycin, clarithromycin, linezolid, mupirocin, doxycycline, minocycline, and tigecycline. The *in vitro* activities observed suggest that antibacterials, which act by inhibiting protein synthesis, are promising candidate therapies for the treatment of pythiosis.

Pythiosis is a life-threatening and chronic pyogranulomatous disease caused by the fungus-like pathogen *Pythium insidiosum*, the main oomycete species capable of infecting humans and other animals (1, 2). Although there have been a few reports of clinical cures of cases of pythiosis with antifungal therapy (3, 4), the data from the literature on the clinical management of pythiosis patients with treatment by antifungals indicate that such therapy has been largely ineffective (2, 5, 6). The genus *Pythium* is unable to synthesize ergosterol, the active target of most antifungals, which partly explains why this class of drugs has been ineffective (2).

Studies on the *in vitro* susceptibility of the clinical isolates of *P. insidiosum* to antifungal drugs have shown divergent results, and there are no international protocols approved for evaluating the *in vitro* susceptibility of *P. insidiosum*. Interestingly, previous studies have shown that *P. insidiosum* is quite sensitive to antibacterials belonging to the classes of macrolides, tetracyclines, and glycyliclones (7, 8) and that its combination with antifungal drugs can result in synergistic interactions *in vitro* (9). However, the effects of antibacterial agents that inhibit protein synthesis on *P. insidiosum* have not been studied extensively. Thus, the objective of this study was to compare the *in vitro* susceptibilities of *P. insidiosum* to a number of antibacterial and antifungal drugs using the Clinical and Laboratory Standards Institute (CLSI) M38-A2 broth microdilution, CLSI M51-A disk diffusion, and Etest methods.

We evaluated the susceptibility of 25 Brazilian *P. insidiosum* strains isolated from equine pythiosis lesions. All of the isolates were identified using a PCR-based assay according to Botton et al. (10). The reference strains included *P. insidiosum* CBS 101555 from an equine pythiosis case and *P. insidiosum* CBS 119452 and *Pythium aphanidermatum* CBS 128995 from human pythiosis cases.

The broth microdilution (BMD) reference assay was carried out following the CLSI M38-A2 guidelines (11), as previously described (12, 13). The final concentrations of the antimicrobial agents tested in the wells ranged from 1,024 to 0.5 µg/ml for mupirocin and tobramycin and from 256 to 0.125 µg/ml for azithromycin, clarithromycin, clindamycin, chloramphenicol, doxycycline, erythromycin, florfenicol, fluconazole, fusidic acid, linezolid, minocycline, roxithromycin, terbinafine, tetracycline, tigecycline, and tilmicosin. The final concentrations for amphotericin B, caspofungin, itraconazole, posaconazole, and voriconazole ranged from 32 to 0.015 µg/ml. All standard anti-

microbial powders were purchased from Sigma-Aldrich (St. Louis, MO, USA). The MIC of each agent was determined by visual observation and represents the inhibition of 100% of mycelium growth after 24 h and 48 h of incubation at 37°C.

Susceptibilities of the isolates to antibacterial and antifungal drugs were also determined by the Etest (AB bioMérieux, Solna, Sweden) method according to the manufacturer's recommendations and by the disk diffusion reference (CLSI M51-A) assay (14). A *P. insidiosum* inoculum at a concentration of 3 to 5 × 10⁴ zoospores/ml was set as the optimal inoculum size for the Etest and disk diffusion assay. Briefly, the entire surface of each 100-mm-diameter nonsupplemented Mueller-Hinton (MH) agar (pH 7.3 ± 0.1) (Hi-Media Laboratories, India) plate was inoculated with 200 µl of the inoculum suspension and spread with a Drigalsky spreader. Excess surface moisture was removed with a sterile pipette. Etest strips (listed in Table 1) and the disks (Sensifar, São Paulo, Brazil) containing 2 µg of clindamycin and lincomycin; 5 µg of mupirocin; 10 µg of gentamicin, neomycin, streptomycin, and tobramycin; 15 µg of azithromycin, clarithromycin, erythromycin, roxithromycin, tigecycline, and tilmicosin; and 30 µg of chloramphenicol, doxycycline, florfenicol, linezolid, minocycline, and tetracycline were placed on the surface of each plate of inoculated agar and incubated for 24 to 48 h at 37°C, after which the zone diameters for disk diffusion testing and the MICs for the Etest strips were recorded. Susceptibility endpoints were read at the intersection of the scale of the strip with the first completely clear ellipse (Etest) and clear inhibition zone (disk diffusion). Off-scale MICs were transformed to the next highest dilution matching the scale for calculating the geometric mean and the essential agreement. The MIC pairs obtained using the BMD and Etest methods were evaluated to either be in essential agreement or show nonsubstantial or substantial differences (15). CLSI and

Received 10 December 2013 Returned for modification 14 May 2014

Accepted 7 September 2014

Published ahead of print 15 September 2014

Address correspondence to Janio M. Santurio, janio.santurio@gmail.com.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.02680-13>.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AAC.02680-13

TABLE 1 *In vitro* susceptibilities of 27 isolates of *Pythium insidiosum* and *Pythium aphanidermatum* CBS 128995 to antibacterials and antifungal agents^d

Antimicrobial agent	Results with CLSI M38-A2 method:			Results with Etest method:			% of isolates showing any discrepancy ^b						Zone diameter by disk diffusion technique ^c (range [mean]) (mm) at:		
	MIC ₉₀ (μg/ml) at:		MIC (range [GM]) (μg/ml) at:	MIC ₉₀ (μg/ml) at:		MIC (range [GM]) (μg/ml) at:	% EA at ^a :		NSD at:		SD at:		24 h	48 h	
	24 h	48 h		24 h	48 h		24 h	48 h	24 h	48 h	24 h	48 h			
Antibacterials															
Azithromycin	8	16	1–8 (2.9)	4	4	0.03–4 (0.7)	0.03–16 (1.0)	78	75	18	18	4	7	14–40 (29.2)	14–40 (27.7)
Chloramphenicol	128	256	4–>256 (23.1)	256	>256	2–>256 (25.6)	8–>256 (53.8)	93	82	3.5	14	3.5	4	NZ–40 (26.3)	NZ–40 (23.8)
Clarithromycin	8	8	0.25–8 (1.8)	8	32	0.5–16 (2.4)	0.5–32 (3.9)	93	93	7	7	0	0	20–38 (28.3)	12–36 (25.4)
Clindamycin	64	256	4–>256 (16)	64	256	2–256 (7.6)	2–>256 (14.5)	82	79	14	17	4	4	NZ–21 (11.5)	NZ–21 (9.4)
Doxycycline	8	16	1–8 (3.3)	4	16	1–8 (2.3)	2–16 (5.8)	100	100	0	0	0	0	22–38 (30.0)	20–38 (29.4)
Erythromycin	32	64	1–32 (7.7)	ND	ND	ND	ND	ND	NA	NA	NA	NA	NA	NZ–34 (22.9)	NZ–34 (21.2)
Fluorfenicol	128	>256	8–>256 (25.1)	ND	ND	ND	ND	ND	NA	NA	NA	NA	NA	NZ–39 (28.6)	NZ–36 (25.6)
Gentamicin	ND	ND	ND	ND	ND	ND	ND	ND	NA	NA	NA	NA	NA	NZ	NZ
Fusidic acid	>256	>256	>256	>256	>256	>256	>256	100	100	0	0	0	0	ND	ND
Linezolid	>256	>256	>256	ND	ND	ND	ND	NA	NA	NA	NA	NA	NA	NZ	NZ
Minocycline	16	32	1–32 (5.6)	4	4	0.5–8 (1.7)	0.5–8 (2.0)	86	75	14	25	0	0	18–46 (31.5)	18–40 (29.3)
Mupirocin	2	4	0.125–4 (0.9)	1	2	0.06–4 (0.2)	0.06–4 (0.4)	82	82	18	18	0	0	21–40 (31.9)	20–40 (31.5)
Neomycin	16	32	2–32 (3.2)	2	4	0.125–2 (0.6)	0.125–4 (1.0)	75	79	21	18	4	3	20–32 (25.8)	16–32 (23.6)
Roxithromycin	ND	ND	ND	ND	ND	ND	ND	ND	NA	NA	NA	NA	NA	NZ	NZ
Streptomycin	32	128	2–128 (9.7)	ND	ND	ND	ND	ND	NA	NA	NA	NA	NA	10–34 (18.9)	NZ–30 (14.6)
Synercid	ND	ND	ND	ND	ND	ND	ND	ND	NA	NA	NA	NA	NA	NZ	NZ
Tetracycline	16	32	1–32 (7.4)	>32	>32	0.5–>32 (5.8)	0.5–>32 (6.9)	NA	NA	NA	NA	NA	NA	ND	ND
Tigecycline	4	4	0.25–4 (1.3)	ND	ND	ND	ND	NA	NA	NA	NA	NA	NA	11–42 (27.4)	10–38 (25.1)
Tilmicosin	128	128	4–128 (27.6)	1	1	0.03–4 (0.2)	0.03–4 (0.3)	68	64	32	32	0	4	23–40 (32.2)	22–40 (33.4)
Tobramycin	>1,024	>1,024	>1,024	ND	ND	ND	ND	NA	NA	NA	NA	NA	NA	NZ–28 (17.6)	NZ–28 (15.2)
Antifungals															
Amphotericin B	>32	>32	>32	>32	>32	>32	>32	100	100	0	0	0	0	ND	ND
Anidulafungin	ND	ND	ND	>32	>32	>32	>32	NA	NA	NA	NA	NA	NA	ND	ND
Caspofungin	>32	>32	16–>32 (47.5)	>32	>32	4–>32 (39.0)	4–>32 (46.3)	96	96	4	4	0	0	ND	ND
Fluconazole	>256	>256	>256	>256	>256	>256	>256	100	100	0	0	0	0	ND	ND
Micafungin	ND	ND	ND	>32	>32	>32	>32	NA	NA	NA	NA	NA	NA	ND	ND
Itraconazole	>32	>32	>32	>32	>32	>32	>32	100	100	0	0	0	0	ND	ND
Posaconazole	>32	>32	>32	>32	>32	>32	>32	100	100	0	0	0	0	ND	ND
Terbinafine	16	32	2–16 (8.0)	ND	ND	ND	ND	NA	NA	NA	NA	NA	NA	ND	ND
Voriconazole	>32	>32	>32	>32	>32	>32	>32	100	100	0	0	0	0	ND	ND

^a Essential agreement (EA) (discrepancies of no more than ± 2 -fold dilutions) between the CLSI and Etest methods.^b Nonsubstantial differences (NSD) (discrepancies of 3 or 4 2-fold dilutions) or substantial differences (SD) (discrepancies of ≥ 4 2-fold dilutions) with respect to the BMD method.^c Not performed for *P. insidiosum* CBS 101555, *P. insidiosum* CBS 119452, or *P. aphanidermatum* CBS 128995 control strains.^d Abbreviations: GM, geometric mean; ND, not determined; NA, not applicable; NZ, no zone.

Etest MIC₉₀s (MICs at which 90% of isolates were inhibited) were also determined.

Table 1 summarizes the *in vitro* susceptibility of the *Pythium* isolates determined using the BMD, disk diffusion, and Etest methods. Tigecycline, minocycline, azithromycin, and clarithromycin were considered the most effective drugs tested because they required the lowest concentrations (geometric mean MICs of <4 µg/ml) for the inhibition of the *Pythium* species. For all strains, the lowest MIC₉₀ values (≤4 µg/ml after 48 h) were observed for tigecycline, minocycline, azithromycin, linezolid, and mupirocin using the BDM and the Etest methods. Lower essential agreements (64 to 86%) were observed for azithromycin, chloramphenicol, clindamycin, linezolid, minocycline, mupirocin, and tigecycline. Excellent essential agreements (93 to 100%) were observed for the other MIC comparisons.

The growth of *Pythium* spp. was not inhibited by amphotericin B, anidulafungin (Etest), micafungin (Etest), itraconazole, posaconazole, or voriconazole (MICs, >32 µg/ml), fluconazole, fusidic acid, or lincomycin (MICs, >256 µg/ml), or tobramycin (MICs, >1,024 µg/ml). Inhibition zones were observed for all of the selected disks with the exception of lincomycin and the aminoglycoside antimicrobials. After 48 h, the highest ranges in halos (zone diameter range in mm [arithmetic mean in mm]) were observed for azithromycin (14 to 40 [27.7]), clarithromycin (12 to 36 [25.4]), linezolid (18 to 40 [29.3]), mupirocin (16 to 32 [23.6]), doxycycline (20 to 38 [29.4]), minocycline (20 to 40 [31.5]), tetracycline (10 to 38 [25.1]), and tigecycline (22 to 40 [33.3]).

In this study, we describe antibacterial drugs that inhibit the *in vitro* growth of *P. insidiosum* at concentrations 100 times lower than those observed for previously studied antifungal drugs. It is noteworthy that antibacterial drugs such as azithromycin, minocycline, and tigecycline can inhibit the growth of *P. insidiosum* isolates *in vitro* at concentrations of <1 µg/ml, while the MICs for amphotericin B, echinocandins, and triazole antifungals are >32 µg/ml.

Previous studies have determined the *in vitro* susceptibility of *P. insidiosum* to antifungal and antibacterial agents using three main assays. In the first of these assays, antimicrobial agents are added to the culture medium, and the changes in the radial growth (16) or the mycelium weight (17) are measured. The second assay, the broth macrodilution or microdilution technique, uses a hyphal suspension scraped from the culture medium, which is adjusted spectrophotometrically (13, 18). The third method, macrodilution (12, 19–21) or microdilution (7, 8, 22, 23) in broth, utilizes the CLSI M38-A2 protocols (11) and inocula with final concentrations of 2 to 3 × 10³ zoospores/ml. Additionally, some studies have reported that *P. insidiosum* isolates were insensitive or resistant to all of the antimicrobials evaluated (24–27) or described the MICs obtained by *in vitro* susceptibility tests (3, 4, 28) without describing how the inocula were prepared or how the susceptibility test was performed. This diversity in susceptibility testing techniques likely correlates with the diversity of MICs observed for *P. insidiosum* (see Table S2 in the supplemental material). The phylogenetic diversity, especially among the clinical isolates from the Americas and Asia (29–31), may be another factor that is related to the different susceptibility profiles of *P. insidiosum*.

This study represents the first assessment of *P. insidiosum* susceptibility using the disk diffusion technique based on the CLSI

M51-A method as well as the first comparison of antimicrobial susceptibility testing between the Etest and CLSI M38-A2 methods. The established pharmacology and safety of the antibacterials described in this work support their consideration as new therapeutic options for the treatment of pythiosis infections. However, it is important to note that *in vivo* experimental testing of the disease in animal models is needed to determine the actual therapeutic potential of these drugs and their associations with treatments by other antimicrobial agents before they can be used safely in the clinic.

In conclusion, the *in vitro* data reported here suggest that antibacterial drugs that act by inhibiting bacterial protein synthesis merit attention as new candidates for the treatment of pythiosis infections. Therefore, additional studies using geographically and genetically diverse *P. insidiosum* strains, in addition to *in vitro* and *in vivo* susceptibility studies with combinations of antimicrobials, are needed for a better understanding of the susceptibility of this species to the antibacterials described in this study.

ACKNOWLEDGMENTS

This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (CAPES-AUX PE-PNPD 743/2012). E.S.L. is a Ph.D. fellow of Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (PNPD-CAPES).

REFERENCES

- Mendoza L, Vilela R. 2013. The mammalian pathogenic oomycetes. *Curr. Fungal Infect. Rep.* 7:198–208. <http://dx.doi.org/10.1007/s12281-013-0144-z>.
- Gaastera W, Lipman LJA, De Cock AWAM, Exel TK, Pegge RBG, Scheurwater J, Vilela R, Mendoza L. 2010. *Pythium insidiosum*: an overview. *Vet. Microbiol.* 146:1–16. <http://dx.doi.org/10.1016/j.vetmic.2010.07.019>.
- Heath JA, Kiehn TE, Brown AE, LaQuaglia MP, Steinhertz LJ, Bearman G, Wong M, Steinhertz PG. 2002. *Pythium insidiosum* pleuropneumonia complicating pneumonia in a child with leukemia. *Clin. Infect. Dis.* 35: E60–E64. <http://dx.doi.org/10.1086/342303>.
- Shenep JL, English BK, Kaufman L, Pearson TA, Thompson JW, Kaufman RA, Frisch G, Rinaldi MG. 1998. Successful medical therapy for deeply invasive facial infection due to *Pythium insidiosum* in a child. *Clin. Infect. Dis.* 27:1388–1393. <http://dx.doi.org/10.1086/515042>.
- Krajaeun T, Sathapatayavongs B, Prachartam R, Nitiyanant P, Leelachaikul P, Wanachiwanawin W, Chairasert A, Assanasen P, Saipetch M, Mootsikapun P, Chetchotisakd P, Lekhakula A, Mitarnun W, Kalnauwakul S, Supparatpinyo K, Chaiwarith R, Chiewchanvit S, Tananuvat N, Srisiri S, Suankratay C, Kulwichit W, Wongsaisuan M, Somkaew S. 2006. Clinical and epidemiological analyses of human pythiosis in Thailand. *Clin. Infect. Dis.* 43:569–576. <http://dx.doi.org/10.1086/506353>.
- Sathapatayavongs B, Leelachaikul P, Prachartam R, Atichartakarn V, Sriphojanart S, Trairatvorakul P, Jirasiritham S, Nontasut S, Eurvilaichit C, Flegel T. 1989. Human pythiosis associated with thalassemia hemoglobinopathy syndrome. *J. Infect. Dis.* 159:274–280. <http://dx.doi.org/10.1093/infdis/159.2.274>.
- Mahl DL, de Jesus FPK, Loreto ES, Zanette RA, Ferreira L, Ben Pilotto M, Alves SH, Santurio JM. 2012. *In vitro* susceptibility of *Pythium insidiosum* isolates to aminoglycoside antibiotics and tigecycline. *Antimicrob. Agents Chemother.* 56:4021–4023. <http://dx.doi.org/10.1128/AAC.00073-12>.
- Loreto ES, Mario DAN, Denardi LB, Alves SH, Santurio JM. 2011. *In vitro* susceptibility of *Pythium insidiosum* to macrolides and tetracycline antibiotics. *Antimicrob. Agents Chemother.* 55:3588–3590. <http://dx.doi.org/10.1128/AAC.01586-10>.
- Jesus FP, Ferreira L, Loreto ES, Pilotto MB, Ludwig A, Bizzi K, Tondolo JS, Zanette RA, Alves SH, Santurio JM. 2014. *In vitro* synergism obtained by azithromycin, clarithromycin, minocycline or tigecycline associates with antifungal agents against *Pythium insidi-*

- osum. Antimicrob. Agents Chemother. 58:5621–5625. <http://dx.doi.org/10.1128/AAC.02349-02314>.
10. Botton SA, Pereira DIB, Costa MM, Azevedo MI, Argenta JS, Jesus FPK, Alves SH, Santurio JM. 2011. Identification of *Pythium insidiosum* by nested PCR in cutaneous lesions of Brazilian horses and rabbits. Curr. Microbiol. 62:1225–1229. <http://dx.doi.org/10.1007/s00284-010-9781-4>.
11. Clinical and Laboratory Standards Institute. 2008. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard, 2nd ed. CLSI document M38-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
12. Pereira DIB, Santurio JM, Alves SH, Argenta JS, Potter L, Spanemberg A, Ferreira L. 2007. Caspofungin *in vitro* and *in vivo* activity against Brazilian *Pythium insidiosum* strains isolated from animals. J. Antimicrob. Chemother. 60:1168–1171. <http://dx.doi.org/10.1093/jac/dkm332>.
13. Fonseca AO, Pereira DI, Maia Filho FS, Osorio LG, Maroneze BP, Valente JS, Potter L, Meireles MC. 2014. *In vitro* susceptibility of zoospores and hyphae of *Pythium insidiosum* to antifungals. J. Antimicrob. Chemother. 69:1564–1567. <http://dx.doi.org/10.1093/jac/dku021>.
14. Clinical Laboratory Standards Institute. 2010. Reference method for antifungal disk diffusion susceptibility testing of non-dermatophyte filamentous fungi; approved guideline. CLSI document M51-A. Clinical and Laboratory Standards Institute, Wayne, PA.
15. Cuenca-Estrella M, Gomez-Lopez A, Alastruey-Izquierdo A, Bernal-Martinez L, Cuesta I, Buitrago MJ, Rodriguez-Tudela JL. 2010. Comparison of the Vitek 2 antifungal susceptibility system with the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) Broth Microdilution Reference Methods and with the Sensititre YeastOne and Etest techniques for *in vitro* detection of antifungal resistance in yeast isolates. J. Clin. Microbiol. 48:1782–1786. <http://dx.doi.org/10.1128/JCM.02316-09>.
16. Brown TA, Grooters AM, Hosgood GL. 2008. *In vitro* susceptibility of *Pythium insidiosum* and a *Lagenidium* sp to itraconazole, posaconazole, voriconazole, terbinafine, caspofungin, and mefenoxam. Am. J. Vet. Res. 69:1463–1468. <http://dx.doi.org/10.2460/ajvr.69.11.1463>.
17. McMeekin D, Mendoza L. 2000. *In vitro* effect of streptomycin on clinical isolates of *Pythium insidiosum*. Mycologia 92:371–373. <http://dx.doi.org/10.2307/3761492>.
18. Sekhon AS, Padhye AA, Garg AK. 1992. *In vitro* sensitivity of *Penicillium marneffe* and *Pythium insidiosum* to various antifungal agents. Eur. J. Epidemiol. 8:427–432. <http://dx.doi.org/10.1007/BF00158578>.
19. Cavalheiro AS, Maboni G, de Azevedo MI, Argenta JS, Pereira DIB, Spader TB, Alves SH, Santurio JM. 2009. *In vitro* activity of terbinafine combined with caspofungin and azoles against *Pythium insidiosum*. Antimicrob. Agents Chemother. 53:2136–2138. <http://dx.doi.org/10.1128/AAC.01506-08>.
20. Cavalheiro AS, Zanette RA, Spader TB, Lovato L, Azevedo MI, Botton S, Alves SH, Santurio JM. 2009. *In vitro* activity of terbinafine associated to amphotericin B, fluvastatin, rifampicin, metronidazole and ibuprofen against *Pythium insidiosum*. Vet. Microbiol. 137:408–411. <http://dx.doi.org/10.1016/j.vetmic.2009.01.036>.
21. Argenta JS, Santurio JM, Alves SH, Pereira DIB, Cavalheiro AS, Spanemberg A, Ferreira L. 2008. *In vitro* activities of voriconazole, itraconazole, and terbinafine alone or in combination against *Pythium insidiosum* isolates from Brazil. Antimicrob. Agents Chemother. 52:767–769. <http://dx.doi.org/10.1128/AAC.01075-07>.
22. Argenta JS, Alves SH, Silveira F, Maboni G, Zanette RA, Cavalheiro AS, Pereira PL, Pereira DIB, Sallis ESV, Potter L, Santurio JM, Ferreira L. 2012. *In vitro* and *in vivo* susceptibility of two-drug and three-drug combinations of terbinafine, itraconazole, caspofungin, ibuprofen and fluvastatin against *Pythium insidiosum*. Vet. Microbiol. 157:137–142. <http://dx.doi.org/10.1016/j.vetmic.2011.12.003>.
23. Argenta JS, Alves SH, Silveira F, Maboni G, Pereira DIB, Spanemberg A, Santurio JM, Ferreira L. 2010. *In vitro* paradoxical growth of *Pythium insidiosum* in the presence of caspofungin. Vet. Microbiol. 145:321–323. <http://dx.doi.org/10.1016/j.vetmic.2010.04.001>.
24. Krockenberger MB, Swinney G, Martin P, Rothwell TRL, Malik R. 2011. Sequential opportunistic infections in two German Shepherd dogs. Aust. Vet. J. 89:9–14. <http://dx.doi.org/10.1111/j.1751-0813.2010.00666.x>.
25. Schloemer NJ, Lincoln AH, Mikhailov TA, Collins CL, Di Rocco JR, Kehl SC, Chusid MJ. 2013. Fatal disseminated *Pythium insidiosum* infection in a child with Diamond-Blackfan anemia. Infect. Dis. Clin. Pract. 21:e24–e26. <http://dx.doi.org/10.1097/IPC.0b013e318278f3b5>.
26. Mendoza L, Prasla SH, Ajello L. 2004. Orbital pythiosis: a non-fungal disease mimicking orbital mycotic infections, with a retrospective review of the literature. Mycoses 47:14–23. <http://dx.doi.org/10.1046/j.1439-0507.2003.00950.x>.
27. Murdoch D, Parr D. 1997. *Pythium insidiosum* keratitis. Aust. N. Z. J. Ophthalmol. 25:177–179. <http://dx.doi.org/10.1111/j.1442-9071.1997.tb01304.x>.
28. Hoffman MA, Cornish NE, Simonsen KA. 2011. A painful thigh lesion in an immunocompromised 11-year-old boy. Pediatr. Infect. Dis. J. 30:1011–1018. <http://dx.doi.org/10.1097/INF.0b013e318225b327>.
29. Azevedo MI, Botton SA, Pereira DIB, Robe LJ, Jesus FPK, Mahl CD, Costa MM, Alves SH, Santurio JM. 2012. Phylogenetic relationships of Brazilian isolates of *Pythium insidiosum* based on ITS rDNA and cytochrome oxidase II gene sequences. Vet. Microbiol. 159:141–148. <http://dx.doi.org/10.1016/j.vetmic.2012.03.030>.
30. Schurko AM, Mendoza L, Levesque CA, Desautels NL, De Cock WAM, Klassen GR. 2003. A molecular phylogeny of *Pythium insidiosum*. Mycol. Res. 107:537–544. <http://dx.doi.org/10.1017/S0953756203007718>.
31. Kammarnjesadaku P, Palaga T, Sritunyalucksana K, Mendoza L, Krajaeun T, Vanittanakom N, Tongchusak S, Denduangboripant J, Chindamporn A. 2011. Phylogenetic analysis of *Pythium insidiosum* Thai strains using cytochrome oxidase II (COX II) DNA coding sequences and internal transcribed spacer regions (ITS). Med. Mycol. 49:289–295. <http://dx.doi.org/10.3109/13693786.2010.511282>.