Review of methods and antimicrobial agents for susceptibility testing against *Pythium insidiosum*

Hanna Yolanda a,b, Theerapong Krajaejun c,*

a Section for Translational Medicine, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand
b Department of Parasitology, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, Jakarta, Indonesia
c Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand

**ABSTRACT**

Pythiosis is a life-threatening infectious disease of humans and animals caused by the oomycete microorganism *Pythium insidiosum*. The disease has been increasingly diagnosed worldwide. *P. insidiosum* inhabits freshwater and presents in two forms: mycelium and zoospore. Clinical manifestations of pythiosis include an infection of the artery, eye, skin, or gastrointestinal tract. The management of pythiosis is problematic due to the lack of effective treatment. Many patients die from an uncontrolled infection. The drug susceptibility testing provides clinically-useful information that could lead to proper drug selection against *P. insidiosum*. Currently, no standard CLSI protocol for the drug susceptibility of *P. insidiosum* is available. This review aims at describing methods and antimicrobial agents for susceptibility testing against *P. insidiosum*. Several in-house in vitro susceptibility methods (i.e., broth microdilution method, radial growth method, and agar diffusion method) have been established for *P. insidiosum*. Either mycelium or zoospore can be an inoculum. Rabbit is the commonly-used model of pythiosis for in vivo drug susceptibility testing. Based on the susceptibility results (i.e., minimal inhibitory concentration and inhibition zone), several antibacterial and antifungal drugs, alone or combination, exhibited an in vitro or in vivo effect against *P. insidiosum*. Some distinct compounds, antiseptic agents, essential oils, and plant extracts, also show anti-*P. insidiosum* activities. Successfully medical treatment, guided by the drug susceptibility data, has been reported in some pythiosis patients. Future studies should emphasize finding a novel and effective anti-*P. insidiosum* drug, standardizing in vitro susceptibility method and correlating drug susceptibility data and clinical outcome of pythiosis patients for a better interpretation of the susceptibility results.

1. Introduction

Pythiosis is a life-threatening infectious disease of humans and animals caused by the oomycete microorganism *Pythium insidiosum* [1, 2, 3]. Pythiosis has been increasingly diagnosed worldwide [4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25]. The disease affects various mammals, predominantly humans [6, 7, 8, 9, 10, 19, 20], horses [14, 18, 26, 27], and dogs [21, 28, 29]. *P. insidiosum* inhabits freshwater and presents in two forms: mycelium and zoospore [30, 31, 32]. Clinical manifestations of pythiosis include an infection of artery, eye, skin, or gastrointestinal tract [33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50]. Pythiosis exhibits high morbidity and mortality rates [7, 8, 28, 49, 51]. Early diagnosis and prompt treatment are critical factors to determine the favorable outcome of an affected individual. The diagnosis of pythiosis relies on clinical presentation and laboratory investigations [10, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68]. The use of conventional antifungal drugs is usually ineffective against *P. insidiosum* due to the lack of a drug-target ergosterol biosynthesis pathway [31, 69]. The main treatment of pythiosis in humans and animals (including equine, the most affected species) relies on extensive surgical intervention [14, 26, 70, 71, 72, 73, 74, 75]. Such treatment is expensive and could lead to postsurgical complications and life-long disability. Many patients die from an uncontrolled infection [76, 77, 78]. A more effective treatment is urgently needed for pythiosis.

Many investigators have searched for a chemical that is capable of inhibiting *P. insidiosum* [79, 80, 81, 82, 83, 84, 85]. Although a standardized susceptibility method for *P. insidiosum* is not available, several in vitro and in vivo assays have been proposed to evaluate drugs against the pathogen [86, 87, 88, 89]. Here, we summarized recent advances in

---

* Corresponding author.
E-mail address: mr_en@hotmail.com (T. Krajaejun).

https://doi.org/10.1016/j.heliyon.2020.e03737
Received 1 December 2019; Received in revised form 30 January 2020; Accepted 31 March 2020

2405-8440/© 2020 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
anti-*P. insidiosum* agents and the in-house susceptibility methods for testing them. Such information could facilitate the selection of the most suitable and effective drug for the treatment of pythiosis. This work was approved by the Committee for Research, Faculty of Medicine Ramathibodi Hospital, Mahidol University (Approval numbers: MURA2019/713, MURA2019/1227, and MURA2020/291).

2. Drug susceptibility testing against *P. insidiosum*

2.1. Inoculum preparation

2.1.1. Zoospores

Zoospore is an infective stage of *P. insidiosum* and colonizes on a water plant. Upon exposure to a human or animal host, the zoospore attaches and germinates as hyphae into affected tissue [31]. Zoospores can be prepared in a laboratory and used as an inoculum for *in vitro* or *in vivo* susceptibility testing against *P. insidiosum* [79, 86, 90, 91]. The method for the production of zoospores is described in detail elsewhere [91, 92]. Briefly, *P. insidiosum* is induced to produce zoospores by co-incubation with sterile grass leaves (i.e., *Paspalum notatum*) on 2% water agar (pH 6.9) at 37 °C for 24 h. *P. insidiosum*-colonizing grass leaves are immersed in the induction medium. After incubation at 37 °C for a few hours, a zoosporangium, containing up to 40 mobile biflagellate zoospores, can be observed under a microscope [92]. Released zoospores can swim ~25 min before encystment [92]. The zoospores are collected, counted by a Neubauer chamber [91, 93], and adjusted to 2–3 x 10^3 cells/ml in RPMI for a drug susceptibility assay [86, 90].

2.1.2. Mycelia

Two types of mycelial inoculum can be prepared: hyphal suspension and agar plugs. For the hyphal suspension, *P. insidiosum* is subcultured on 0.1% yeast extract agar and incubated at 37 °C for four days [94]. The colony is then scraped using a sterile scalpel blade in the presence of 10 ml sterile distilled water [94, 95]. The obtained hyphal suspension is adjusted to 80–85% transmittance at the 530-nm wavelength. The hyphal suspension is diluted (1:10) in the Roswell Park Memorial Institute (RPMI) 1640 broth before using it as inoculum in the broth microdilution method [94, 95]. For the hyphal plug, *P. insidiosum* is grown on Sabouraud dextrose agar (SDA) at 37 °C for five days [69, 82]. An agar plug (5 mm in diameter) is excised from the edge of an actively-growing colony and used as inoculum in the radial growth method [69, 82, 87], the agar diffusion method [96, 97, 98, 99, 100, 101], or the broth microdilution method [100, 101, 102, 103, 104].

2.2. *In vitro* drug susceptibility methods

2.2.1. Broth microdilution method

By modifying the Clinical and Laboratory Standards Institute (CLSI) M38-A2 protocol, Pereira et al. used the broth microdilution method for susceptibility testing of *P. insidiosum* zoospores, as summarized in

---

**Figure 1.** Broth microdilution method for susceptibility testing of an antimicrobial agent against *P. insidiosum*. The inoculum (zoospores), medium (RPMI-1640), and drug (in two-fold dilutions) are prepared for co-incubation in a microdilution plate. After incubation at 37 °C for 24 h, minimal inhibitory concentration (MIC), minimal effective concentration (MEC), fractional inhibitory concentration index (FICI) (in case of two-drug combination), and minimal cidal concentration (MCC) can be determined.
Figure 1 [81,105]. The generated zoospores are resuspended in RPMI-1640 broth, adjusted to pH 6.9-7.1 with 0.164 M 3-[N-morpholino] propane sulfonic acid (MOPS) and the final concentration of 2–3 x 10^6 cells/ml [81, 106]. The zoospore suspension (inoculum) is tested against RPMI-1640 alone (no-drug control) and various drug concentrations in a microdilution tray. The tetrazolium salt (a colorimetric indicator) can be added in the drug-zoospore mixture to facilitate the assay interpretation (only the viable P. insidiosum hyphae turns purple) [106]. Minimal inhibitory concentration (MIC) or minimal effective concentration (MEC) is determined after 24-h incubation at 37 °C [81, 105, 106, 107]. MIC is the lowest drug concentration that visually demonstrates 100% growth inhibition [81, 106], whereas MEC is the lowest drug concentration that results in morphological changes of the organism [105, 107]. The minimum cidal concentration (MCC) is the lowest drug concentration that no growth is observed after incubating the drug-treated organism on a drug-free culture agar (i.e., SDA) for up to 96 h at 37 °C [79, 81, 90, 105].

Because the zoospore is challenging to generate in the laboratory, the hyphal agar plug can be alternatively used as the inoculum in the broth microdilution method [102, 103, 104]. The hyphal agar plugs are directly added into drug-containing broth. The susceptibility result is determined by weighing organism-dried weight [104], culturing drug-treated hyphae [100, 101, 103, 108], or directly observing organism growth by the naked eye [102].

To evaluate the effect of two-drug combination (i.e., drugs A and B), a fractional inhibitory concentration index (FICI) score is calculated, using the following formula: FICI = (MIC of drug A in the drug A-B combination/MIC of drug A) + (MIC of drug B in the drug A-B combination/MIC of drug B). The obtained FICI score defines synergistic (FICI <0.5), indifferent (0.5 < FICI ≤ 4), and antagonistic (FICI >4) interactions of the drug combination against P. insidiosum [107, 109].

2.2.2. Radial growth method

Some investigators used the radial growth method to study drug susceptibility against P. insidiosum [69, 82, 87, 102]. Briefly, a hyphal agar plug (as inoculum) is excised from the edge of an actively-growing P. insidiosum colony on SDA (or vegetable extract agar) and placed on a new agar plate containing various drug concentrations (including no-drug control) [69, 82, 87]. The hyphal side of the plug should be in direct contact with the drug-containing agar [82]. The agar plates are incubated at 37 °C for 2–3 days before measuring a colony diameter. The mean colony diameter of each strain is subtracted by the agar plug width (~5 mm) and divided by two to obtain the radial growth of drug-treated P. insidiosum [69, 82, 87, 102]. Radial growth-based MIC is the drug concentration that completely inhibits P. insidiosum growth.

2.2.3. Agar diffusion method

Zoospores [79, 88] and hyphal agar plugs [96, 97, 110, 111] have been used as inoculum in the agar diffusion susceptibility method, as depicted in Figure 2. The number of zoospores used in the agar diffusion method (3–5 x 10^5 cells/ml) is markedly different from that used in the broth microdilution method (2–3 x 10^5 cells/ml) [79, 81, 88, 106]. Approximately 200 μl of the zoospores suspension was spread on the entire surface of a non-supplemented Mueller Hinton (MH) agar plate, and the excess liquid is removed using a sterile pipette [88, 105]. A drug-containing disk is placed on the surface of each plate and incubated for 24–48 h at 37 °C before the measurement of the clear zone diameter [79, 88]. The commercial E-test (bioMérieux, France) or MIC Test Strip (Liofilchem, Italy) can replace the drug-containing disk, and MIC is read from the provided scale [79, 88].

Some natural compounds have been evaluated for their anti-P. insidiosum activities, using the agar diffusion method [96, 97, 98, 99, 100, 101, 110, 111]. A hyphal agar plug (1 × 1 cm in size) is placed in the center of an SDA plate and inoculated at room temperature. Afterward, a disk soaked with 20 μl of the natural compound or extract is put on the same SDA plate, placed 2 cm apart from the hyphal agar plug [112]. The inhibition zone is measured after prolonged incubation for 3–9 days at 25 °C.

2.3. In vivo drug susceptibility method

In vivo susceptibility study is clinically useful for the determination of drug efficacy against P. insidiosum [80, 81, 85, 103]. Rabbit is the commonly-used experimental model of pythiosis for in vivo drug susceptibility analysis [80, 86, 113, 114]. The animals are inoculated subcutaneously with ~2 × 10^4 viable zoospores/ml, and P. insidiosum infection is usually established within 25 days post-inoculation [86, 113]. An antimicrobial agent is then administered in the infected animals [80, 81, 113, 114]. Changes in sizes of the lesion (i.e., subcutaneous nodular area), blood tests, microbiological workups (i.e., culture and PCR), and histopathologic results are used to assess the extent of P. insidiosum infection in response to the tested drug [80, 86, 113].
3. Antimicrobial agents against *P. insidiosum*

Several groups of antimicrobial drugs, such as antifungals, antibiotics, natural extracts, and some other compounds, have been investigated in vitro and in vivo for their anti-*P. insidiosum* effects, as summarized below:

3.1. Antifungal drugs

3.1.1. Allylamines

Terbinafine was designed to inhibit the enzyme squalene epoxidase (ERG1) of the fungal sterol biosynthetic pathway [69]. It has been used in the treatment of pythiosis since its first report on the successful medical treatment of this disease [7, 51, 72, 115, 116, 117, 118, 119, 120]. However, administration of terbinafine, usually in combination with itraconazole, has shown a favorable response in only a few pythiosis patients [7, 72, 117, 120]. MICs of terbinafine varied (range: 0.5–128 μg/ml), depending on *P. insidiosum* strains tested (i.e., different genotypes) and the susceptibility methods used (i.e., broth dilution and radial growth) [69, 72, 86, 87, 88, 95, 107, 109, 117, 121, 122, 123, 124, 125, 126, 127, 132] (Table 1). Because *P. insidiosum* lacks the ERG1-encoding gene [69], it is still mysterious about how terbinafine exhibits antifungal activity against some strains of this pathogen.

3.1.2. Azoles

Azole drugs inhibit fungi by inactivating the 14-α-sterol demethylase (ERG11) [72,122,128–131]. The ERG11-encoding gene is present in *P. insidiosum*, but phylogenetically diverse from that of the true fungi [69]. This finding suggests that *P. insidiosum* ERG11 may not be an optimal target of the azole drugs. The azoles comprise two subclasses: imidazoles (i.e., miconazole and ketoconazole) and triazoles (i.e., itraconazole, voriconazole, fluconazole, and posaconazole). These drugs had diverse *in vitro* antimicrobial activities against *P. insidiosum* (Table 1). For example, MICs of miconazole ranged from 2 to 32 μg/ml, whereas that of ketoconazole ranged between 4 and 64 μg/ml [95, 122]. Compared to imidazoles, triazoles generally exhibited a broader MIC range. MICs of itraconazole were reportedly different from study to study (Table 1), ranging from 1 to >128 μg/ml [69, 72, 85, 86, 87, 88, 95, 107, 117, 121, 122, 123, 125, 126, 127, 132]. Voriconazole and fluconazole had MICs greater than 16 μg/ml [88, 107, 121] and 32 μg/ml [88, 122], respectively, against Brazilian isolates of *P. insidiosum*. In contrast, these two drugs inhibited Thai isolates at MICs lesser than 8 μg/ml [72, 117, 125, 132]. Posaconazole showed anti-*P. insidiosum* activity with MICs greater than 8 μg/ml [87, 88].

3.1.3. Polyenes

The polyene drug, amphotericin B, binds ergosterol in the cell membrane and forms pores that lead to ion leakage and cell death [133]. MICs of amphotericin B, tested against the animal isolates of *P. insidiosum*, were 4–128 μg/ml [88, 107, 123], while tested against the human strains, were 4–8 μg/ml [72, 117, 125, 132] (Table 1). The lack of the endogenous ergosterol (drug target), due to the incomplete ergosterol biosynthesis pathway in *P. insidiosum* [69], could explain clinical unresponsiveness to amphotericin B in some cases [8, 12, 71, 76, 116]. However, in the treatment of several horses with pythiosis, the intravenous regional limb perfusion of amphotericin B, in conjunction with surgical intervention and thermocautery, showed significant

| Drug class | Drug name | Susceptibility method(s) | MIC (μg/ml) | Reference(s) |
|------------|-----------|--------------------------|-------------|--------------|
| Allylamines | Terbinafine | BMD Horses (15–30) Brazil | 0.5–128 8.0–32.0 | [86, 88, 95, 107, 109, 121, 122, 123, 124, 126] |
|            |           | BMD Humans (1–22) Thailand | 2-4 NA | [72, 117, 125, 132] |
|            |           | RGM Dogs (6) USA | >8 >8 | [87] |
|            |           | RGM Humans (30) Thailand | >128 >128 | [69] |
| Azoles     | Miconazole | BMD Horse (17–22) Brazil | 2–32 13.6 | [95, 122] |
|            | Ketoconazole | BMD Horse (17–22) Brazil | 4–64 23.1 | [95, 122] |
|            | Itraconazole | BMD Horse (15–30) Brazil | >16 >16 | [88, 88, 95, 107, 121, 123, 126] |
|            |           | BMD Human (1–22) Thailand | 1–4 NA | [72, 117, 125, 132] |
|            |           | RGM Dog (6) USA | >8 >8 | [87] |
|            |           | RGM Human (30) Thailand | >128 >128 | [69] |
|            | Voriconazole | BMD, ADM Horse (28–30) Brazil | >16 >16 | [88, 107, 121] |
|            |           | BMD Human (1–22) Thailand | 1–8 NA | [72, 117, 125, 132] |
|            |           | RGM Dog (6) USA | >8 >8 | [67] |
|            | Fluconazole | BMD, ADM Horse (17–28) Brazil | >32 59.0 | [88, 122] |
|            |           | BMD Human (1–22) Thailand | 1–8 NA | [72, 117, 125, 132] |
|            | Posaconazole | BMD, ADM Horse (28) Brazil | >32 >32 | [88] |
|            |           | RGM Dog (6) USA | >8 >8 | [67] |
| Polyenes   | Amphotericin B | BMD, ADM Horse (17–30) Brazil | 4–128 25.1–34.3 | [88, 107, 109, 123] |
|            |           | BMD Human (1–22) Thailand | 4–8 NA | [72, 117, 125, 132] |
| Echinocandins | Caspofungin | BMD, ADM Horse (15–30) Brazil | 4–256 16.0–94.8 | [81, 86, 88, 107, 122, 123, 137] |
|            |           | BMD Human (1–22) Thailand | 2–8 NA | [72, 117, 125, 132] |
|            |           | RGM Dog (6) USA | >2 >2 | [87] |
|            | Anidulafungin | BMD, ADM Horse (28–30) Brazil | >32 1000.6 | [88, 107] |
|            |           | BMD Human (1–22) Thailand | 2–8 NA | [72, 117, 125, 132] |
|            | Micafungin | BMD, ADM Horse (17–30) Brazil | >32 776.0 | [88, 107, 114] |
| Others     | Griseofulvin | BMD Human (1) Thailand | >32 >32 | [125] |
|            | 5-Fluorocytosine | BMD Unknown (1) China | 4 4 | [127] |

**Abbreviations:** BMD, broth microdilution method; RGM, radial growth method; ADM, agar diffusion method; MIC, minimal inhibitory concentration; NA, data not available.
regression of the lesion, complete epithelialization, and no sign of recurrence during a one-year follow-up [134, 135].

3.1.4. Echinocandins

Echinocandins (i.e., caspofungin, anidulafungin, and micafungin) were designed to inhibit β-1,3-D-glucan synthase, which forms glucan, a major cell wall component of fungi and oomycetes [31, 136]. Each echinocandin drug had a different anti- P. insidiosum activity (Table 1). When P. insidiosum isolates from horses in Brazil were tested by broth microdilution method, MICs of caspofungin ranged from 4 to 256 μg/ml [81, 86, 88, 107, 122, 123, 137], which were generally lower than MICs of anidulafungin (>32 μg/ml) [88, 107] and micafungin (>32 μg/ml) [88, 107, 114]. This observation suggests that caspofungin is more potent than the other echinocandins tested. As opposed to the horse strains, relatively-lower MICs of caspofungin and anidulafungin (2–8 μg/ml) were observed in the human isolates from Thailand [72, 117, 125, 132]. MEC (the lowest drug concentration that changes the microscopic morphology of the organism) has been used to evaluate the responsiveness of P. insidiosum to echinocandins. Caspofungin exhibited better MECs (8–32 μg/ml), compared to micafungin (16–128 μg/ml) and anidulafungin (>256 μg/ml) [107, 114]. In vivo susceptibility of caspofungin against P. insidiosum in the rabbit model of pythiosis showed a reduced lesion size (i.e., subcutaneous nodule) and a decrease in hyphal burden [81, 113]. However, the subcutaneous lesion regrew when discontinuing caspofungin administration, indicating that the drug had, to some extent, a static microbial effect [81].

3.1.5. Other antifungal drugs

Griseofulvin and 5-fluorocytosine, classified in two separated groups of antifungals, were also tested against P. insidiosum (Table 1). Regarding the mechanism of action, griseofulvin disrupts the microtubule function and the assembly of the mitotic spindle, while 5-fluorocytosine inhibits thymidylate synthetase and impairs DNA synthesis [131]. Griseofulvin had both microdilution-based MIC of >32 μg/ml [125], whereas 5-fluorocytosine showed such MIC of 4 μg/ml [127] (Table 1).

3.2. Antibacterial drugs

Different classes of systemic and topical antibacterial drugs have been evaluated for their in vitro or in vivo anti-P. insidiosum activities. Compared with the antifungals, some antibacterial drugs exhibited a relatively-greater inhibitory activity against P. insidiosum. Recent reports on the susceptibility of P. insidiosum to the antibacterial drugs are summarized below and in Table 2.

3.2.1. Tetracyclines and glycyclines

Tetracyclines inhibit protein synthesis by binding the 30S bacterial ribosomal subunit and blocking the access of aminocyl-tRNA to the mRNA-ribosome complex [138]. Based on the broth microdilution method, minocycline, doxycycline, tetracycline, and oxytetracycline can differentially suppress P. insidiosum growth at MICs of 0.02–4 μg/ml [9, 80, 88, 106, 107, 123, 125, 139], 0.13–16 μg/ml [9, 88, 106, 125, 139], 0.19–32 μg/ml [9, 88, 106, 140], and 2–32 μg/ml [106], respectively (Table 2). Based on the agar diffusion method, the minimum inhibition zones of P. insidiosum were not markedly different: 28.7–31.9 mm for minocycline, 22.3–30 mm for doxycycline, and 23.7–27.4 mm for tetracycline [5, 88, 141] (Table 2). In vivo susceptibility information of minocycline showed a 17% cure rate in the rabbit model of pythiosis [80]. Glycylcycines are synthetic analogs of the tetracyclines and share the mechanism of action [142]. Tigecycline, a derivative of minocycline, had MICs of 0.02–4 μg/ml [9, 80, 88, 90, 107, 123, 139] and the mean inhibition zones of 27.2–32.2 mm [9, 88] against P. insidiosum (Table 2). In vivo evaluation of tigecycline showed an increased lung invasion of P. insidiosum in one out of six experimental rabbits with pythiosis [80].

3.2.2. Macrolides

Macrolides suppress the peptidyl transferase or block the ribosome exit tunnel of a nascent peptide [143]. Broth microdilution-based MIC ranges of the macrolide drugs against P. insidiosum have been reported (Table 2): clarithromycin, 0.05–64 μg/ml; azithromycin, 0.02–32 μg/ml; erythromycin, 1–32 μg/ml; roxithromycin, 2–128 μg/ml; josamycin, 2–64 μg/ml; and tilmicosin, 4–128 μg/ml [9, 79, 86, 88, 106, 107, 123, 124, 125, 139, 140]. The agar diffusion method with macrolides demonstrated different inhibition zones of P. insidiosum (Table 2): azithromycin (22.1–29.2 mm), clarithromycin (20.5–28.3 mm), erythromycin (22.9 mm), roxithromycin (18.9 mm), and tilmicosin (17.6 mm) [9, 88, 141]. In vivo susceptibility study in the rabbit model of pythiosis revealed an increased P. insidiosum burden for clarithromycin and an 83% cure rate for azithromycin [80].

3.2.3. Pleuromutins, streptogramins, and lincosamides

The antibacterial mechanism of pleuromutins, streptogramins, and lincosamides is similar to that of macrolides. They inhibit peptidyl transferase in the large ribosomal subunit or interfere with polypeptide elongation [143, 144, 145, 146]. The broth microdilution-based MICs of these drug classes against P. insidiosum were summarized in Table 2. MIC ranges of the pleuromutins were 0.25–32 μg/ml for repetapamulin, 0.25–16 μg/ml for valnemulin, and 2–64 μg/ml for tiamulin [79]. MICs of the combination of quinupristin and dalfopristin ranged from 0.5 to >32 μg/ml [88, 140]. MICs of the lincosamides were from 2 to >256 μg/ml for clindamycin [88, 140] and >256 μg/ml for lincomycin [88]. Information on the mean inhibition zones of P. insidiosum (based on agar diffusion method) was available for two drugs: clindamycin (11.5 mm) and lincomycin (no inhibition zone) [88].

3.2.4. Oxazolidinones

Oxazolidinones bind P site of the 50S ribosomal subunit and prevent the formation of a large ribosomal-Met-tRNA complex that initiates protein synthesis [145]. The oxazolidinones can suppress the growths of P. insidiosum at MICs of 0.5–64 μg/ml for linezolid, 4–64 μg/ml for sutezolid, and >32 μg/ml for tedizolid [9, 79, 88, 125, 139, 140] (Table 2). Linezolid showed the mean inhibition zone of 31.2–31.5 mm [9, 88, 141] (Table 2).

3.2.5. Phenics

Phenics (also known as amphenicols) prevent the binding of the aminocyl tRNA to the 50S bacterial ribosomal subunit and inhibit protein synthesis [145, 146, 147]. By the broth microdilution method, MICs of florfenicol and chloramphenicol, against P. insidiosum, were in the range of 2 to >256 μg/ml [9, 88, 140]. The agar diffusion method, using these two drugs, showed the mean inhibition zones in the range of 12.2–28.6 mm [9, 88, 141] (Table 2).

3.2.6. Aminoglycosides

Aminoglycosides bind polypeptides and interfere with protein synthesis by causing misreading and premature termination of mRNA translation [145]. In general, aminoglycosides, such as paromomycin, streptomycin, gentamicin, neomycin, tobramycin, kanamycin, and amikacin, had anti-P. insidiosum effect at broth microdilution-based MICs of >4 μg/ml (Table 2) [88, 90, 125, 139, 140]. Some investigators demonstrated that these drugs could exhibit MICs of up to 64 μg/ml [90]. Besides, half of P. insidiosum isolates tested had a significant reduction in dry weight after exposed to streptomycin [104]. Based on the agar diffusion method, aminoglycosides showed no inhibition zone of P. insidiosum [88, 141].

3.2.7. Other antibacterial drugs

Rifampicin, metronidazole, and nitrofurantoin have been evaluated for anti-P. insidiosum activities, using the broth microdilution method (Table 2). MICs of these drugs were diverse: for example, >2 μg/ml for rifampicin, from 32 to 128 μg/ml for metronidazole, and from 64 to >64 μg/ml for nitrofurantoin [109, 140]. P. insidiosum has been tested against
| Drug class | Drug name       | P. insidiosum | MIC (μg/ml) | Inhibition zone (mm) | References |
|------------|----------------|--------------|-------------|----------------------|------------|
|            |                | Host (number of isolates) | Country of origin | Range | Mean | Range | Mean | |
| Tetracyclines | Minocycline  | Horse (25–30) | Brazil | 0.06–4 | 0.2–1.0 | 21–40 | 31.9 | [80, 88, 106, 107, 123] |
|             |                | Horse (11) | USA, Costa Rica | 0.25–4 | 1.1–2.0 | NA | NA | [139] |
|             |                | Human (38–48) | India | 0.02–4 | 0.6 | 18–35 | 28.7 | [9] |
|             |                | Human (1) | Japan | NA | NA | Large inhibition zone | [141] |
|             |                | Human (1–27) | Thailand | 1–4 | 1.6–2.0 | NA | NA | [125, 139] |
|             |                | Environment (12) | Thailand | 2–4 | 2.0–2.2 | NA | NA | [139] |
| Doxycycline | Horse (26–28) | Brazil | 0.5–8 | 1.8–3.3 | 22–38 | 30 | [88, 106] |
|             |                | Horse (11) | USA, Costa Rica | 1–16 | 3.4–4.0 | NA | NA | [139] |
|             |                | Human (38–48) | India | 0.13–12 | 3.1 | 14–32 | 22.3 | [9] |
|             |                | Human (1–27) | Thailand | 1–16 | 3.7–4.3 | NA | NA | [125, 139] |
|             |                | Environment (12) | Thailand | 2–16 | 4.0–4.8 | NA | NA | [139] |
| Tetracycline | Horse (25–28) | Brazil | 1–32 | 6.0–8.7 | 11–42 | 27.4 | [88, 106, 140] |
|             |                | Human (38–48) | India | 0.19–24 | 5.09 | 16–34 | 23.7 | [9] |
| Macrolides  | Clarithromycin | Horse (25–30) | Brazil | 0.25–64 | 1.4–4.5 | 20–38 | 28.3 | [79, 80, 88, 106, 107, 123] |
|             |                | Horse (11) | USA, Costa Rica | 0.13–2 | 1.0–1.4 | NA | NA | [139] |
|             |                | Human (38–48) | India | 0.05–4 | 1.7 | 6–34 | 20.5 | [9] |
|             |                | Human (1–27) | Thailand | 0.13–8 | 0.5–1.7 | NA | NA | [125, 139] |
|             |                | Environment (12) | Thailand | 1–4 | 1.2–1.6 | NA | NA | [139] |
| Azithromycin | Horse (21–30) | Brazil | 0.03–32 | 0.7–6.9 | 14–40 | 29.2 | [79, 80, 88, 106, 107, 123, 124] |
|             |                | Horse (11) | USA, Costa Rica | 2–8 | 2.7–2.8 | NA | NA | [139] |
|             |                | Human (38–48) | India | 0.02–32 | 5.4 | 6–33 | 22.1 | [9] |
|             |                | Human (1) | Japan | NA | NA | Intermediate inhibition zone | [141] |
|             |                | Human (1–27) | Thailand | 1–16 | 3.1–5.3 | NA | NA | [125, 139] |
|             |                | Environment (12) | Thailand | 2–16 | 4.0–4.8 | NA | NA | [139] |
| Erythromycin | Horse (25–28) | Brazil | 1–32 | 6.4–7.7 | 0–34 | 22.9 | [88, 106, 140] |
|             |                | Human (1) | Japan | NA | NA | Intermediate inhibition zone | [141] |
|             |                | Horse (28) | Brazil | 2–128 | 9.7 | 10–34 | 18.9 | [88] |
|             |                | Josamycin | Horse (30) | 2–64 | 16 | NA | NA | [79] |
|             |                | Tilmicosin | Horse (28) | 4–128 | 27.6 | 0–28 | 17.6 | [88] |
| Pleuromutins | Retapamulin | Horse (30) | Brazil | 0.25–32 | 1.45 | NA | NA | [79] |
|             |                | Valnemulin | Horse (30) | 0.25–16 | 2.09 | NA | NA | [79] |
|             |                | Tiamulin | Horse (30) | 2–64 | 16.4 | NA | NA | [79] |
| Streptogramins | Quinupristin and Dalfopristin | Horse (25–28) | Brazil | 0.5–>32 | 2.8–5.8 | NA | NA | [88, 140] |
| Lincosamides | Clindamycin | Horse (25–28) | Brazil | 2–>256 | 7.0–16.0 | 0–21 | 11.5 | [88, 140] |
| Lincomycin | Horse (28) | Brazil | >256–256 | No inhibition zone | [88] |
| Oxazolidinones | Linezolid | Horse (25–30) | Brazil | 0.5–64 | 1.7–13.3 | 18–46 | 31.5 | [79, 88, 140] |
|             |                | Horse (11) | USA, Costa Rica | 4–8 | 5.4–8.0 | NA | NA | [139] |
|             |                | Human (38–48) | India | 0.75–32 | 7.7 | 20–44 | 31.2 | [9] |
|             |                | Human (1) | Japan | NA | NA | Large inhibition zone | [141] |
|             |                | Human (1–27) | Thailand | 4–32 | 8.0–9.2 | NA | NA | [125, 139] |
|             |                | Environment (12) | Thailand | 4–16 | 9.5 | NA | NA | [139] |
| Sutezolid | Horse (30) | Brazil | 4–64 | 7.5 | NA | NA | [79] |
|             |                | Tazedolid | Horse (30) | >32 | >32 | NA | NA | [79] |
| Phenics | Florfenicol | Horse (28) | Brazil | 8–>256 | 25.1 | 0–39 | 28.6 | [88] |
| Chloramphenicol | Horse (25–28) | Brazil | 2–>256 | 23.1–27.1 | 0–40 | 26.3 | [88, 140] |
|             |                | Human (38–48) | India | 16–256 | 204.6 | 6–25 | 12.2 | [9] |
|             |                | Human (1) | Japan | NA | NA | Intermediate inhibition zone | [141] |

(continued on next page)
some other drugs, such as fusidic acid (MIC >256 μg/ml), daptomycin (>4 μg/ml), novobiocin (>1.6 μg/ml), optochin (concentration not defined), quinolones (>4 μg/ml), vancomycin (>16 μg/ml), bacitracin (concentration not defined), trimethoprim-sulfamethoxazole (>2–38 μg/ml), polymyxins (≥8 μg/ml), carbapenems (>4 μg/ml), penicillins (>8 μg/ml), and cephalosporins (>2 μg/ml) [88, 125, 139, 140, 141].

3.2.8. Topical antimicrobial drugs
Several topical antiseptics showed antimicrobial activities against *P. insidiosum*. Most of the topical antimicrobials tested (i.e., triclosan, mupirocin, cetylpyridinium chloride, benzalkonium chloride, and cetrimide) had MICs less than 32 μg/ml [9, 88, 124, 140]. Crystal violet completely inhibited the growths of all *P. insidiosum* isolates studied [140]. No anti-*P. insidiosum* activity was observed with potassium permanganate at the maximal concentration tested (64 μg/ml) [124].

3.3. Natural extracts

3.3.1. Plant-extracted essential oils
Plant-extracted essential oils from *Origanum vulgare*, *Origanum majorana*, *Mentha piperita*, *Rosmarinus officinalis*, and *Melaleuca alternifolia* have shown in vitro antimicrobial effect against *P. insidiosum* (Table 3). For example, *O. vulgare*-derived oil mainly consisted of carvacrol (71–93%), possessed MICs of 50–1,750 μg/ml [94, 126, 148], and the purified carvacrol had MICs of 80–320 μg/ml [123]. The extracted oils from *O. majorana* (containing 34% of 4-terpineol), *M. piperita* (30–58% of menthone) and *R. officinalis* (65% of 1,8-cineole) demonstrated MICs of 50–3,500 μg/ml, 110–3,500 μg/ml, and 110–3, 500 μg/ml, respectively [94, 126, 148]. *M. alternifolia* oil (containing 40–52% of terpinene-4-ol) exhibited MICs of 531–2,125 μg/ml [94, 126, 149]. Similarly, nanoemulsion (mixed with 1% of *M. alternifolia* oil) showed MICs of 133–2,125 μg/ml [149].

A combination of *M. piperita* and *O. vulgare* oils had synergized antimicrobial effects against 65% of *P. insidiosum* isolates tested [94]. However, *M. alternifolia* oil, combined with either *M. piperita* or *O. vulgare* oil, showed no additional anti-*P. insidiosum* activity [94]. A mixture of the antifungal drug itraconazole (but not terbinfine) and either *M. alternifolia*, *M. piperita*, or *O. vulgare* oil increased the inhibitory effect on 60–95% of the recruited *P. insidiosum* isolates [126]. When *O. vulgare* and *M. piperita* oil were topically applied, in conjunction with *P. insidiosum* antigen administration (so-called immunotherapy), the skin lesion in the rabbit model of pythiosis was relatively smaller, compared with applying each oil alone [83].

3.3.2. Plant-extracted compounds
Some compounds extracted from the plants using ethyl acetate and methanol can suppress *P. insidiosum* growths (Table 3). For instance, isomicrocimel, micromarin B, 7-methoxy-8-(4′-methyl-3′-furanyl) coumarin, and secomicrocimel were derived from *Micromelum falcatum* fruit and at the concentration of 0.20–0.22 mM, showed *P. insidiosum*-

| Drug class | Drug name      | Host (number of isolates) | Country of origin | MIC (μg/ml)a | Inhibition zone (mm)b | References |
|------------|----------------|---------------------------|-------------------|--------------|----------------------|-----------|
| Aminoglycosides | Paromomycin | Horse (11) | USA, Costa Rica | 16–32 | 26.9–32 | NA NA | 139 |
| Streptomycin | Horse (24–28) | Brazil | 32–64 | 50.7 | No inhibition zone | 68, 90 |
| Gentamicin | Horse (24–28) | Brazil | >8 | 55.3 | No inhibition zone | 88, 90, 140 |
| Neomycin | Horse (11) | USA, Costa Rica | 32–32 | 32–32 | NA NA | 139 |
| Tobramycin | Horse (11) | USA, Costa Rica | >8 | >8 | No inhibition zone | 88, 140 |
| Rifampicin | Horse (17–25) | Brazil | >2 | 61.4 | NA NA | 109, 140 |
| Metronidazole | Horse (17) | Brazil | 32–128 | 66.6 | NA | 109 |
| Nitrofurantoin | Horse (25) | Brazil | 64–>64 | 105.4 | NA | 140 |

Abbreviations: MIC, minimal inhibitory concentration; NA, data not available.

a Minimal inhibitory concentration measured by broth microdilution method and agar diffusion method (E-test and MIC test strip).

b Inhibition zone measured by agar diffusion method (Disk diffusion).
inhibited zones of 6.2–21 mm [97]. Alyxia schlechteri root-extracted pinosinol, alyterinate C, and medioresinol (at the concentration of 65–76 μg/ml) affected P. insidiosum growths by showing the inhibition zones of 13.3–16.1 mm [110]. Likewise, the clausine K, zapoterin, clausine L, and N-methylswietenidine B (extracted from Clausena harmandiana root; at the concentration of 1,000 μg/ml) or greater antimicrobial effect against P. insidiosum [96, 100, 101], and it was not toxic to fibroblast cell lines [100]. The vestilol, trihydroxy chalcone, dihydromaackiain, mucronulatol, dalpulanone, and duartin extracted from Dalbergia stipulacea stem (concentration: 1,000 μg/ml) showed 9.8, 5.1, 7.7, 6.6, 6.7, and 4.2 mm inhibition zones against P. insidiosum, respectively [99].

The aqueous phase alcohol extract of the garlic Allium sativum (mainly composed of allicin) had the anti-P. insidiosum MIC of <6,250 μg/ml [150] (Table 3). The methanol extract of Stryphnodendron adstringens bark (containing 46% of tannin) showed the minimal cidal concentrations (MCC) of 1,000–1,500 μg/ml against P. insidiosum growth, while the purified tannin possessed lower MCCs (<1,000 μg/ml) [103] (Table 3). The scanning electron microscopy demonstrated an altered cell wall of the tannin-treated P. insidiosum [103]. Nevertheless, either extracted or commercial tannin failed to recover the experimental rabbits with pythiosis [103].

### 3.3.3. Other natural compounds

Bees produce propolis and geopropolis that exhibit antimicrobial activities [108]. These natural substances were ethanol extracted from the selected bees and used to explore the anti-P. insidiosum effect [108]. The extracted propolis (from Africanized honeybees) and geopropolis (from Melipona stingless bee) had MCCs of 3.4 and 12.5 mg/ml, respectively (Table 3). Approximately 10 μl of synthetic volatile organic compounds of the endophytic fungus Mascodor crispans (strain B23) can completely suppress the growths of all P. insidiosum isolates tested [82].

| Source of compound | Identified compound(s) | P. insidiosum | Reference(s) |
|--------------------|-----------------------|---------------|--------------|
| **Host** (number of isolates) | **Country of origin** | **MIC (μg/ml)** | **Inhibition zone (mm)** |
| **Originum vulgare oil** | Carvacrol | Horse (20-22) | Brazil | 50-1,750 | NA | [94, 126, 140] |
| **Purified carvacrol** | Carvacrol | Horse (25) | Brazil | 80–320 | NA | [123] |
| **Originum majorana oil** | 4-terpineol | Horse (22) | Brazil | 50-3,500 | NA | [148] |
| **Mentha piperita oil** | Menthone | Horse (20-22) | Brazil | 110-3,500 | NA | [94, 126, 148] |
| **Rosmarinus officinalis oil** | 1,8-cineole | Horse (22) | Brazil | 110-3,500 | NA | [148] |
| **Melaleuca alternifolia oil** | Terpinene-4-ol | Horse (20-26) | Brazil | 133-2,125 | NA | [94, 126, 149] |
| **Micromelum falcatum (fruit)** | Isomicroomelin | Unknown (1) | Thailand | NA | 21.0 (0.22 mM) | [97] |
| | Micromarin B | Unknown (1) | Thailand | NA | 19.2 (0.21 mM) | [97] |
| | 7-methoxy-8-(4-methyl-3-furanyl)coumarin | Unknown (1) | Thailand | NA | 15.5 (0.20 mM) | [97] |
| | Secomicroomelin | Unknown (1) | Thailand | NA | 6.2 (0.22mM) | [97] |
| **Alyxia schlechteri (root)** | Pinoresinol | Unknown (1) | Thailand | NA | 16.1 (76 μg/μl) | [110] |
| | Alyterinate C | Unknown (1) | Thailand | NA | 16.0 (73 μg/μl) | [110] |
| | Medioresinol | Unknown (1) | Thailand | NA | 13.3 (65 μg/μl) | [110] |
| **Clausena harmandiana (root)** | Clausine K | Unknown (1) | Thailand | NA | 16.2 (10 μg/μl) | [111] |
| | Zapoterin | Unknown (1) | Thailand | NA | 11.8 (40 μg/μl) | [111] |
| | Clausine L | Unknown (1) | Thailand | NA | 10.2 (40 μg/μl) | [111] |
| | N-methylswietenidine B | Unknown (1) | Thailand | NA | 7.9 (58 μg/μl) | [111] |
| **Dalbergia stipulacea (stem)** | (-)-vestitol | Human (1) | Thailand | NA | 2.9–9.8 (1-1,000 μg/ml) | [99] |
| | 2',4',4'-trihydroxy chalcone | Human (1) | Thailand | NA | 3.8-5.1 (10-1,000 μg/ml) | [99] |
| | Dihydromaackiain | Human (1) | Thailand | NA | 7.4-7.7 (100-1,000 μg/ml) | [99] |
| | Mucronulatol | Human (1) | Thailand | NA | 5.9-6.6 (100-1,000 μg/ml) | [99] |
| | Dalpulanone | Human (1) | Thailand | NA | 4.9-6.7 (100-1,000 μg/ml) | [99] |
| | Duartin | Human (1) | Thailand | NA | 3.7-4.2 (100-1,000 μg/ml) | [99] |
| **Stryphnodendron adstringens (bark)** | Tannin | Horse (15) | Brazil | 1,000–1,500 | NA | [103] |
| **Purified tannin** | Tannin | Horse (15) | Brazil | 500-1,000 | NA | [103] |
| **Allium sativum** | Allicin | Horse (17) | Brazil | <6,250 | NA | [150] |
| **Africanized honeybees propolis** | Benzoic acid, coumaric acid, caffeic acid, artepillin C, eetc. | Horse (15) | Brazil | 3.4 | NA | [108] |
| **Melipona fasciculata geopropolis** | Tripterpenes, anacardic acid, alkytesorincols, etc. | Horse (15) | Brazil | 12.5 | NA | [108] |
| **Pseudomona sputaeri ST1302** | Fraction number 6 | Unknown (11) | Thailand | 3.13 | NA | [100, 101] |
| **Klebsiella pneumoniae ST2501** | Fraction number 1 | Unknown (11) | Thailand | 1.57-3.13 | NA | [100, 101] |

Abbreviations: MIC, minimal inhibitory concentration; NA, data not available.

- Minimal inhibitory concentration measured by broth microdilution method.
- Inhibition zone measured by agar diffusion method (Disk diffusion).
- Extraction using ethyl acetate and methanol.
- Extraction using methanol.
- Extraction using alcohol.
- Extraction using ethanol.
- Metabolites.
Some bacterial metabolites were reported active against *P. insidiosum*. For example, diketopiperazine and pyrrolnitrin of *Pseudomonas stutzeri* (strain ST1302) can inhibit the pathogen [98]. Besides, the metabolite of *Klebsiella pneumoniae* (strain ST2501) had a relatively-stronger anti-*P. insidiosum* activity than that of *P. stutzeri* [100, 101] (Table 3).

### 3.4. Other anti-*P. insidiosum* substances

The other substances that are not grouped with the drugs mentioned above were evaluated for the inhibition of *P. insidiosum* growths. For example, biogenic silver nanoparticles had an anti-*P. insidiosum* MIC range of 0.06–0.47 μg/ml [84]. The effect of the biogenic silver nanoparticle included the destruction of the cell wall and intracellular organelles. The cytotoxic concentration of the nanoparticle was twice as much compared with its effective concentration. Diphenyl diselenide showed MICs of 0.5–2 μg/ml, and this organoselenium compound temporarily reduced the lesion size in the rabbit model of pythiosis [85]. The agricultural fungicide mefenoxam (at 1 μg/ml) can completely inhibit 90% of *P. insidiosum* isolates tested [86]. Miltelosine is an alkyl-phosphocholine drug that possesses potent antiparasitic and antimicrobial activities, and it can inhibit *P. insidiosum* at MICs of 0.5–64 μg/ml [79, 151]. However, miltelosine showed a favorable response in the rabbit model of pythiosis [151]. Copper acetate and cadmium acetate are metal compounds that exhibited anti-*P. insidiosum* activity with MICs of 4–64 and 16–256 μg/ml, respectively [152].

Drug repurposing is a strategy to use a drug designed for one particular disease in another condition [153, 154]. Such a strategy has been applied to identify some drugs with anti-*P. insidiosum* effect. For instance, disulfiram, designed for the treatment of alcoholism, showed broth microdilution-based MICs of 8–32 μg/ml [102]. Deferasirox is an iron-chelating drug that had anti-*P. insidiosum* property with MICs of 12.5–50 μg/ml [114, 155]. Although deferasirox destroyed the hyphae and minimized the lesion size, it seemed to promote the dissemination of *P. insidiosum* infection [155, 156]. The lipid-controlling drug, fluvastatin, provided the anti-*P. insidiosum* MIC of >16 μg/ml [86, 109]. Ibuprofen, a nonsteroidal anti-inflammatory drug, showed anti-*P. insidiosum* activity with a broad MIC range of 128–2,048 μg/ml [86, 109].

### 3.5. Drug combinations

A combination of different antimicrobial drugs could contribute to a synergistic, indifferent, or antagonistic effect on *P. insidiosum* growth. Such an effect can be determined by using the MIC-based checkerboard technique [157]. The combination of two antifungal drugs, such as terbinafine and either amphotericin B, itraconazole, voriconazole, voriconazole, ketoconazole, miconazole, or caspofungin, resulted in an indifferent anti-*P. insidiosum* activity in 53–100% of the recruited isolates [72, 86, 109, 117, 121, 122, 127, 132]. Combinations of antibacterial drugs from different classes (i.e., glycolycelines, tetracyclines and macrolides) were analyzed for anti-*P. insidiosum* effects *in vitro* [80, 125, 139]. Several combinations showed a favorable susceptibility outcome. For example, minocycline, combined with either tigecycline, azithromycin or clarithromycin had markedly synergistic anti-*P. insidiosum* effects in ~80% of the *P. insidiosum* isolates tested [80, 199]. However, such drug combinations had an anti-*P. insidiosum* effect in only 17% (minocycline and clarithromycin), 33% (minocycline and tigecycline), and 67% (minocycline and azithromycin) of the experimental rabbits with pythiosis [80].

The effects of antifungal-antibacterial drug combinations on *in vitro* growths of *P. insidiosum* were also investigated, as summarized in Table 4 [107, 109]. All pairs of the selected antifungal and antibacterial drugs resulted in indifference in 27–94% of the isolates tested. Only a few sets of combined drugs (i.e., itraconazole and minocycline; micafungin and tigecycline or clarithromycin) provided a synergistic effect in ~70% of the analyzed isolates. To a lesser extent, several drug combinations (i.e., itraconazole and clarithromycin; terbinafine and rifampicin) exhibited antagonistic activity in up to 7% of the isolates. In two Thai patients with relapsed or inoperable vascular pythiosis, a combination of an antifungal drug (itraconazole or voriconazole) and a few antibacterial agents (i.e., doxycycline, azithromycin, or clarithromycin) can suppress the disease progression during the 64-week follow-up [125]. Drug selection and combination reported in these patients were guided by the susceptibility data [125].

Drug combinations of either terbinafine or azithromycin and a topical antimicrobial agent (i.e., potassium permanganate, cetylpiridinium, tri-closan, mupirocin, and benzalkonium) showed indifferent anti-*P. insidiosum* activity in at least 60% of the strains tested [124]. However, drug synergism can be observed in 71% of the analyzed *P. insidiosum* isolates, if terbinafine was combined with the topical drug cetrimide [124]. When combined with itraconazole, clarithromycin, azithromycin, minocycline, or tigecycline, either carbavcol or thymol (found in plant-extracted oil) had a synergistic outcome in most (60–96%) of the studied isolates [123].

Combinations of antimicrobial and repurposed drugs have shown additionally anti-*P. insidiosum* activities *in vitro* [86, 109, 114, 123]. Micafungin, combined with deferasirox, showed a synergistic effect in 88% of the tested isolates [114]. A three-drug combination of terbinafine, itraconazole, caspofungin, fluvastatin, and ibuprofen demonstrated an indifferent antimicrobial activity in 53–86% of the isolates [86]. The terbinafine-itraconazole-fluvastatin combination showed decreased hyphae burden in the rabbits with pythiosis [86]. However, prominent antagonistic drug interaction was observed in 35% of *P. insidiosum* isolates when the terbinafine-fluvastatin combination was tested [109]. Caution should be raised when using a certain drug combination in vivo, such as terbinafine and caspofungin [86], and micafungin and

*Table 4. In vitro susceptibility testing of the combinations of antifungal (i.e., terbinafine, amphotericin B, itraconazole, voriconazole, caspofungin, anidulafungin, and micafungin) and antibacterial (i.e., minocycline, tigecycline, azithromycin, clarithromycin, metronidazole, rifampicin) drugs against *P. insidiosum*.*

| Drugs | Terbinafine | Amphotericin B | Itraconazole | Voriconazole | Caspofungin | Anidulafungin | Micafungin |
|-------|-------------|----------------|--------------|--------------|-------------|---------------|-----------|
| Minocycline | 63:37:00 | 73:27:00 | 70:30:00 | 60:40:00 | 46:47:07 | 43:57:00 | 63:37:00 |
| Tigecycline | 60:40:00 | 57:43:00 | 47:53:00 | 40:62:00 | 47:53:00 | 43:53:04 | 73:27:00 |
| Azithromycin | 63:37:00 | 40:57:03 | 36:67:03 | 53:47:00 | 43:53:04 | 43:53:04 | 67:33:00 |
| Clarithromycin | 63:37:00 | 63:37:00 | 43:50:07 | 57:43:00 | 53:43:03 | 47:50:03 | 70:30:00 |
| Metronidazole | 06:94:00 | NA:94:NA | NA | NA | NA | NA | NA |
| Rifampicin | 00:94:06 | NA | NA | NA | NA | NA | NA |

- Data were summarized from [107, 109].
- Combinations of metronidazole (or rifampicin) and other drugs were tested against 17 isolates, whereas the other drug combinations were tested against 30 isolates.
- Susceptibility interpretation of Echinocandins (i.e., caspofungin, anidulafungin, and micafungin) was based on Minimal Effective Concentration (MEC).
- Abbreviation: NA, data not available.
deferasirox [114], since such combinations might promote disseminated pythiosis, seen in the rabbit model.

4. Prospective and conclusion

The management of pythiosis is challenging, and in most cases, relies on combined treatment modalities: antimicrobial drugs, surgical intervention, and immunotherapy [7, 8, 49]. While radical surgery could aim at a cure of pythiosis, it leads to disabilities. In some humans and animals with advanced disease, surgical intervention is impossible or provides an unfavorable outcome. The efficacy of the immunotherapy alone, particularly in human patients with pythiosis, has not been evaluated clearly [7, 71, 72, 73, 118, 119]. A handful of conventional antifungal and antibacterial drugs possessed a prominent in vitro anti-P. insidiosum effect (Tables 1 and 2). Some antifungal and antibacterial drugs can decrease P. insidiosum burden and increase the survival rate in the animal model [80, 81, 89, 113]. The synergized anti-P. insidiosum effect has been observed when several drugs were combined [80, 86, 125, 139]. The use of some drugs, such as tigecycline [80], clarithromycin [80] and deferasirox [114, 155], could increase P. insidiosum burden and promote disseminated infection in the experimental rabbits. These possible outcomes should be considered when using such drugs clinically against P. insidiosum.

Drug selection and combination could be guided by in vitro susceptibility testing against the patient isolate of P. insidiosum. For example, co-administration of iraconazole and terbinfine showed the best in vitro anti-P. insidiosum effect, and significantly improved the condition of an American patient with invasive pythiosis without surgical intervention [120]. Two Thai vascular pythiosis patients with the inoperable disease can be controlled, during a long follow-up period (over a year), by administering several antifungal and antibacterial drugs [125]. Besides, there are reports of the successful medical treatment in two Indian and Japanese patients with ocular pythiosis, using the combination of the topical and oral antimicrobial drugs [141, 158, 159]. Some dogs survived intestinal pythiosis after the treatment with corticosteroid and a terbinafine-itraconazole combination, without surgery [160]. These success stories on the management of pythiosis emphasize the clinical usefulness of the in vitro and in vivo susceptibility data. The standard CLSI guideline is not available for in vitro drug susceptibility testing against P. insidiosum. Several in-house susceptibility methods (including broth microdilution method, radial growth method, and agar diffusion method) have been introduced to feasibly assess anti-P. insidiosum effect of various drugs and substances. Inoculum can be prepared from the zoospores or hyphae of P. insidiosum. Selection of a suitable susceptibility method and inoculum type depends on the nature of the substance used, availability of required reagents, skilled personal and objective of the experiment. Interpretation of in vitro susceptibility results (i.e., MIC, inhibition zone) needs to be evaluated clinically to establish a guideline on drug selection and combination. In vivo drug evaluation in an animal model can provide more insight into drug action against P. insidiosum since it demonstrates not only the direct pathogen-drug interaction (as does in vitro assay) but also pharmacokinetic and pharmacodynamic properties of the drug. So far, the rabbit is the primary animal model of pythiosis that has been used for in vivo susceptibility analysis. However, the experimental rabbits with pythiosis usually manifest as a subcutaneous lesion, which does not represent the clinical features of pythiosis in humans and animals [31]. Recently, a mouse model of pythiosis has been developed, and it shows similar clinical features of vascular and disseminated pythiosis observed in humans [93]. Thus, the mouse is an alternative animal model for in vivo drug susceptibility testing against P. insidiosum.

In conclusion, the management of pythiosis is problematic due to the lack of effective treatment. The drug susceptibility testing provides clinically-useful information that can lead to proper drug selection and combination against P. insidiosum. Based on the susceptibility results, several antibacterial and antifungal drugs exhibited a profound anti-P. insidiosum effect. Some distinct compounds, antiseptic agents, essential oils, and plant extracts, have shown anti-P. insidiosum effect. Future studies should emphasize finding a novel and effective anti-P. insidiosum drug, standardizing in vitro susceptibility method, as well as correlating drug susceptibility data and clinical outcome of pythiosis patients for a better interpretation and application of the susceptibility results.

Declarations

Author contribution statement

All authors listed have significantly contributed to the development and the writing of this article.

Funding statement

This work was supported by Faculty of Graduate Studies, Mahidol University, Thailand (H. Yolanda); Section for Translational Medicine, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Thailand (H. Yolanda); School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, Indonesia (H. Yolanda); Thailand Research Fund, Thailand (Grant numbers: RSA6280092 [T. Krajaejun]); and Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Thailand (Grant number: CF 61007 [T. Krajaejun]).

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

[1] A. De Cock, L. Mendoza, A. Padhye, L. Ajello, L. Kaufman, Pythium insidiosum sp. nov., the etiologic agent of pythiosis, J. Clin. Microbiol. 25 (1987) 344–349.
[2] L. Mendoza, R. Vilera, The mammalian pathogenic oomycetes, Curr. Fungal Infect. Rep. 7 (2013) 198–208.
[3] G.W. Beakes, S.L. Glockling, S. Sekimoto, The evolutionary phylogeny of the oomycete ‘fungi’, Prototaxa 249 (2012) 3–19.
[4] I. Tabosa, F. Riet-Correa, V. Nobre, E. Aviedo, J. Reis-Junior, R. Medeiros, Outbreaks of pythiosis in two flocks of sheep in northeastern Brazil, Vet. Pathol. 41 (2004) 412–415.
[5] G. Konradt, D.M. Bassuino, M.V. Bianchi, L. Castro, R.A. Capriolo, S.P. Pavarini, et al., Cutaneous pythiosis in calves: an epidemiologic, pathologic, serologic and molecular characterization, Med. Mycol. Case Rep. 14 (2016) 24–26.
[6] N. Prasertwitayakki, W. Louthrenoo, N. Kastinon, K. Thamprasert, N. Vanitathanakom, Human pythiosis, a rare cause of arteritic case report and literature review, Semin. Arthritis Rheum. 33 (2003) 204–214.
[7] M.N. Chitsazomlot, N. Larbcharoensub, A. Chindampong, T. Krajaejun, Clinico pathological features and outcomes of pythiosis, Int. J. Infect. Dis. 71 (2018) 33–41.
[8] T. Krajaejun, B. Sathapatayavongs, R. Prachatkarn, P. Nityanant, P. Leelachakul, W. Wanachawsanwinit, et al., Clinical and epidemiological analyses of human pythiosis in Thailand, Clin. Infect. Dis. 43 (2006) 569–576.
[9] B. Bagga, S. Sharma, S.J. Guda, R. Nagpal, J. Joseph, K. Manjulatha, et al., Leep forward in the treatment of Pythium insidiosum keratitis, Br. J. Ophthalmol. 102 (2018) 1629–1633.
[10] S. Sharma, P.K. Balne, S.R. Motukapally, S. Das, P. Garg, S.K. Sahu, et al., Pythium insidiosum keratitis: clinical profile and role of DNA sequencing and zoospora formation in diagnosis, Cornea 34 (2015) 438–442.
[11] H. He, H. Liu, X. Chen, J. Wu, M. He, X. Zhong, Diagnosis and treatment of pythium insidiosum corneal ulcer in a Chinese child: a case report and literature review, Am. J. Case Rep. 17 (2016) 982–988.
[12] T.Y. Tanheho, R.C. Stacy, L. Mendoza, M.L. Durand, F.A. Jakobiec, K.A. Colby, Pythium insidiosum keratitis in Israel, Eye Contact Lens 37 (2011) 96–98.
[13] I.S. Barequet, F. Lavinsky, M. Rosner, Long-term follow-up after successful treatment of Pythium insidiosum keratitis in Israel, Semin. Ophthalmol. 28 (2013) 247–250.
[14] Y. Salas, A. Márquez, J. Canelón, Y. Perazzo, V. Colmenarejos, J. López, Equine pythiosis: report in crossed bred (Criole Venezuelan) horses, Mycopathologia 174 (2018) 267–276.
K.M. Thieman, K.A. Kirkby, A. Flynn-Lurie, A.M. Grooters, N.J. Bacon, K.A. Liljebjelke, C. Abramson, C. Brockus, C.E. Greene, Duodenal obstruction in an immunocompromised 11-year-old boy, Pediatr. Infect. Dis. J. 30 (2011) 1011–1017.

S.I. Connolly, C. Frank, C.A. Thompson, W.G. Van Alstine, H. Gelb, H.G. Herg et al., Dual infection with Pythium insidiosum and Blastomyces dermatitidis in a dog, Vet. Clin. Pathol. 41 (2012) 419–423.

C. Hung, D. Leddin, Keratitis caused by Pythium insidiosum in an immunocompromised patient with Crohn’s disease, Clin. Gastroenterol. Hepatol. 12 (2014) A21–A22.

F. Aeffner, M.J. Hall, B.M. Pressler, K.L. Townsend, T.L. Papenfuss, Pathology in ocular pythiosis, Can. J. Ophthalmol. 53 (2018) e48–e50.

K. Neufeld, C. Seamone, B. Maleki, J.G. Heathcote, Pythium insidiosum keratitis: a pictorial essay of natural history, Can. J. Ophthalmol. 137 (2004) 372.

J.R. Fischer, L.W. Face, J.R. Turk, J.M. Kreeger, M.A. Miller, H.S. Gosner, Gastrointestinal insidiosum in Missouri dogs: eleven cases, J. Vet. Diagn. Invest. 6 (1994) 380–382.

J.M. Desautels, J.R. Tomas, L. Almeida, J. Furtado, C. Alves, Gastrointestinal pythiosis in a goat, J. Comp. Pathol. 167 (2017) 215–234.

P. Carro, R. Portela, T. Silva, J. Oliveira-Filho, F. Riet-Correa, Cutaneous pythiosis in a goat, J. Comp. Pathol. 152 (2015) 103–105.

K.M. Thieman, K.A. Kirkby, A. Flynn-Lurie, A.M. Grooters, N.J. Bacon, Gastric pythiosis in a dog, Rev. Iberoam. De. Micol. 29 (2012) 235–237.

T. Krajaejun, T. Rujirawat, C. Srichunrusami, P. Onpeaw, et al., Detection of the oomycete Pythium insidiosum by real-time PCR targeting the gene coding for exo-1,3-β-glucanase, J. Med. Microbiol. 64 (2015) 1891–1892.

A. Krajetjarut, T. Lohnoo, W. Yingyong, T. Rujirawat, C. Srichunrusami, P. Onpeaw, et al., PCR amplification of a putative gene for exo-1,3-β-glucanase to identify the pathogenic oomycete Pythium insidiosum, J. Med. Microbiol. 64 (2015) 91–97.

A. Krajetjarut, T. Lohnoo, W. Yingyong, T. Rujirawat, C. Srichunrusami, P. Onpeaw, et al., Evolution of the sterol biosynthetic pathway of Pythium insidiosum and related oomycetes, J. Biomed. Sci. 25 (2017) 1–11.

M.R. McGinnis, et al., Systemic Pythium insidiosum in a pediatric burn patient, Burns 36 (2010) e68–71.

N. Sermthanaphan, P. Praditnarak, K. Hongku, C. Wongwat, K. Chinsakchai, K. Ruangsetakit, et al., Outcomes and factors influencing prognosis in patients with vascular pythiosis, J. Vasc. Surg. 64 (2011) 411–417.

A.M. Grooters, A. Whittington, M.K. Lopez, M.N. Borroughs, A.F. Roy, Evaluation of microbacterial culture techniques for the isolation of Pythium insidiosum from equine tissues, J. Vet. Diagn. Invest. 14 (2002) 288–294.

T. Krajaejun, P. Chongtrakool, K. Angkananukul, T.T. Brandthoff, Effect of teniposide on growth of the pathogenic oomycete Pythium insidiosum, Southeast Asian J. Trop. Med. Public Health 41 (2010) 1462–1466.

A. Krajetjarut, T. Somprach, T. Chattanonpoom, P. Karonsonth, T. Lerktharith, B. Kunthapat, et al., Protein A/G-based immunochromatographic test for serodiagnosis of pythiosis in human and animal subjects from Asia and America, Sabouraudia 54 (2016) 641–647.

T. Krajaejun, S. Imkhioe, A. Intaramat, K. Ratanaabangkon, Development of an immunochromatographic test for rapid serodiagnosis of human pythiosis, Clin. Vaccine Immunol. 16 (2009) 506–509.

T. Jindayok, S. Prionomsittikorn, S. Srimuang, K. Khuphasu, T. Krajaejun, Hemaggulutination test for rapid serodiagnosis of human pythiosis, Clin. Vaccine Immunol. 16 (2009) 1047–1051.

T. Krajetjarut, P. Karonsonth, R. Aroonwich, S. Srimuang, T. Sangrachi, L. Sansopha, et al., Evaluation of an in-house immunoperoxidase staining assay for histodiagnosis of human pythiosis, Southeast Asian J. Trop. Med. Public Health 40 (2009) 1298–1305.

R. Inkomolue, N. Larcharoenroong, P. Karonsonth, T. Lerktharith, B. Kunthapat, T. Lohnoo, et al., Development of an anti-elicitin antibody-based immunohistochemical assay for diagnosis of pythiosis, J. Clin. Microbiol. 54 (2016) 43–48.

T. Rujirawat, T. Sriplu, T. Lohnoo, W. Yingyong, Y. Kumsang, P. Sae-Chew, et al., Single nucleotide polymorphism-based multiplex PCR for identification and genotyping of the oomycete Pythium insidiosum from humans, animals and the environment, Infect. Genet. Evol. 54 (2017) 429–436.

A. Krajetjarut, T. Lohnoo, W. Yingyong, T. Rujirawat, C. Srichunrusami, et al., Assessment of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification and biotyping of the pathogenic oomycete Pythium insidiosum, J. Med. Microbiol. 64 (2015) 637–644.

A.M. Grooters, M.K. Gee, Development of a nested polymerase chain reaction assay for the detection and identification of Pythium insidiosum, J. Vet. Intern. Med. 16 (2002) 147–152.

P. Konrirkvongs, A. Chaiprasert, P. Uparatankit, M. Chocharoen, R. Banyong, T. Krajaejun, et al., Systemic Pythium insidiosum in patients with suspected fungal keratitis, Southeast Asian J. Trop. Med. Public Health 45 (2014) 167–173.

A.M. Grooters, M.K. Gee, Development of a nested polymerase chain reaction assay for the detection and identification of Pythium insidiosum, J. Vet. Intern. Med. 16 (2002) 147–152.

P. Konrirkvongs, A. Chaiprasert, P. Uparatankit, M. Chocharoen, R. Banyong, T. Krajaejun, et al., Systemic Pythium insidiosum in pathological specimens from patients with suspected fungal keratitis, Southeast Asian J. Trop. Med. Public Health 45 (2014) 167–173.

M.N. Chitasombat, P. Jongkhajornpong, K. Lekhanont, T. Krajaejun, Recent update in diagnosis and treatment of human pythiosis, PeerJ (2020), e8555.

K. Chinsakchai, C. Ruangsetakit, J. Srinarin, Novel duplex polymerase chain reaction for the rapid detection of Pythium insidiosum directly from corneal specimens of patients with ocular pythiosis, Cornea (2020) (in press).

C. Jaturapatrararak, F. Payattikul, T. Lohnoo, Y. Kumsang, A. Laikul, W. Pathsomsudkul, et al., Protein A/G-based enzyme-linked immunosorbent assay for detection of anti-Pythium insidiosum antibodies in human and animal subjects, BMC Res. Notes 10 (2017) 1305.

T. Lerktharith, A. Sircakun, T. Lohnoo, W. Yingyong, T. Rujirawat, T. Krajaejun, Evolution of the sterol biosynthetic pathway of Pythium insidiosum and related oomycetes contributes to antifungal drug resistance, Antimicrob. Agents Chemother. 61 (2017) e02352-16.

M. Kirzhner, S.R. Arnold, C. Lyle, L.L. Mendoza, J.C. Fleming, Pythium insidiosum: a rare necrotizing orbital and facial infection, J. Pediatr. Infect. Dis. Soc. 4 (2014) 155–157.

K. Lekhanont, V. Chackpavong, P. Chongkrakool, R. Aroonwich, A. Vongkhongri, Pythium insidiosum keratitis in contact lens wear: a case report, Cornea 28 (2009) 1173–1177.

G. Wongsitik, W. Pathomsudkul, C. Sorriklin, T. Krajaejun, W. Suthiwitsayahu Phong, First confirmed case of nasal pythiosis in a horse in Thailand, JMM Case Rep. 5 (2018), e005136.
A. S. Tondolo, J. S. Tondolo, T. S. Luz, S. H. Alves, J. M. Santurio, et al., Detection of diketopiperazine and pyrrolnitrin, compounds with anti-
Pythium insidiosum activity, in a Pseudomonas stutzeri environmental strain, Antimicrob. Agents Chemother. 53 (2009) 2136–2140.

A. S. Tondolo, J. S. Tondolo, T. S. Luz, S. H. Alves, J. M. Santurio, et al., Adjunctive antibacterial agents as a salvage therapy in
Pythium insidiosum, J. Clin. Infect. Dis. 27 (1998) 1388–1393.

A. S. Tondolo, J. S. Tondolo, S. H. Alves, J. M. Santurio, et al., In vitro activity of terbinafine associated to amphotericin B, fluvastatin, rifampicin, metronidazole and iberprofen against Pythium insidiosum, Vet. Microbiol. 137 (2009) 408–411.

A. S. Tondolo, J. S. Tondolo, T. S. Luz, S. H. Alves, J. M. Santurio, et al., In vitro activity of terbinafine alone or in combination against Pythium insidiosum isolates from Brazil, Antimicrob. Agents Chemother. 59 (2015) 91–94.

A. S. Tondolo, J. S. Tondolo, T. S. Luz, S. H. Alves, J. M. Santurio, et al., In vitro activities of voriconazole, itraconazole, and
terbinafine and topical antimicrobial treatments against Pythium insidiosum, J. Mycol. Med. 25 (2015) 91–94.

A. S. Tondolo, J. S. Tondolo, T. S. Luz, S. H. Alves, J. M. Santurio, et al., In vitro activity of terbinafine and topical antimicrobial treatments against Pythium insidiosum, Mycol. Med. 19 (2015) 2138–2143.

A. S. Tondolo, J. S. Tondolo, T. S. Luz, S. H. Alves, J. M. Santurio, et al., In vitro susceptibility of Pythium insidiosum isolates to macrolides and tetracycline antibiotics, Antimicrob. Agents Chemother. 53 (2009) 2136–2140.

A. T. Brown, A. D. Green, J. H. Pan, S. P. Kerkar, M. P. Siegenthaler, M. Hughes, P. K. Pandalai, A complicated case of vascular Pythium insidiosum, Antimicrob. Agents Chemother. 53 (2009) 2136–2140.

A. Zanetti, F. Jesus, M. Pilotto, C. Weiblen, L. P. Ferrerio, et al., In vitro activity of terbinafine alone or in combination against Pythium insidiosum, Vet. Microbiol. 104 (2004) 67–72.

A. Zanetti, F. Jesus, M. Pilotto, C. Weiblen, L. P. Ferrerio, et al., Topical terbinafine associated to amphotericin B, fluvastatin and rifampicin, prevents in vitro growth of the oomycete, Vet. Microbiol. 123 (2007) 43–49.

A. Zanetti, F. Jesus, M. Pilotto, C. Weiblen, L. P. Ferrerio, et al., In vitro activity of terbinafine associated to amphotericin B, fluvastatin, rifampicin, metronidazole and iberprofen against Pythium insidiosum, Vet. Microbiol. 157 (2012) 137–142.

A. Zanetti, F. Jesus, M. Pilotto, C. Weiblen, L. P. Ferrerio, et al., In vitro activity of terbinafine alone or in combination against Pythium insidiosum, Vet. Microbiol. 157 (2012) 137–142.

A. Zanetti, F. Jesus, M. Pilotto, C. Weiblen, L. P. Ferrerio, et al., In vitro activity of terbinafine alone or in combination against Pythium insidiosum, Vet. Microbiol. 157 (2012) 137–142.

A. Zanetti, F. Jesus, M. Pilotto, C. Weiblen, L. P. Ferrerio, et al., In vitro activity of terbinafine alone or in combination against Pythium insidiosum, Vet. Microbiol. 157 (2012) 137–142.

A. Zanetti, F. Jesus, M. Pilotto, C. Weiblen, L. P. Ferrerio, et al., In vitro activity of terbinafine alone or in combination against Pythium insidiosum, Vet. Microbiol. 157 (2012) 137–142.

A. Zanetti, F. Jesus, M. Pilotto, C. Weiblen, L. P. Ferrerio, et al., In vitro activity of terbinafine alone or in combination against Pythium insidiosum, Vet. Microbiol. 157 (2012) 137–142.

A. Zanetti, F. Jesus, M. Pilotto, C. Weiblen, L. P. Ferrerio, et al., In vitro activity of terbinafine alone or in combination against Pythium insidiosum, Vet. Microbiol. 157 (2012) 137–142.

A. Zanetti, F. Jesus, M. Pilotto, C. Weiblen, L. P. Ferrerio, et al., In vitro activity of terbinafine alone or in combination against Pythium insidiosum, Vet. Microbiol. 157 (2012) 137–142.

A. Zanetti, F. Jesus, M. Pilotto, C. Weiblen, L. P. Ferrerio, et al., In vitro activity of terbinafine alone or in combination against Pythium insidiosum, Vet. Microbiol. 157 (2012) 137–142.

A. Zanetti, F. Jesus, M. Pilotto, C. Weiblen, L. P. Ferrerio, et al., In vitro activity of terbinafine alone or in combination against Pythium insidiosum, Vet. Microbiol. 157 (2012) 137–142.
