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SUSCEPTIBILITY

In Vitro Susceptibility of Thai *Pythium insidiosum* Isolates to Antibacterial Agents

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ABSTRACT Human pythiosis is a life-threatening human disease caused by *Pythium insidiosum*. In Thailand, vascular pythiosis is the most common form and carries a mortality rate of 10 to 40%, despite aggressive treatment with radical surgery, antifungal agents, and immunotherapy. Itraconazole and terbinafine have been the mainstay of treatment, until recently, based on case report data showing potential synergistic effects against Brazilian *P. insidiosum* isolates. However, the synergistic effects of itraconazole and terbinafine against Thai *P. insidiosum* isolates were not observed. This study tested the *in vitro* susceptibilities of 27 Thai human *P. insidiosum* isolates (clade II, $n = 17$; clade IV, $n = 10$), 12 Thai environmental *P. insidiosum* isolates (clade II, $n = 4$; clade IV, $n = 8$), and 11 non-Thai animal *P. insidiosum* isolates (clade I, $n = 9$; clade II, $n = 2$) to antibiotics in eight antibacterial classes to evaluate alternative effective treatments. Tetracycline and macrolide antibiotics demonstrated *in vitro* activity against Thai *P. insidiosum* isolates, with doxycycline MICs (1 to 16 μg/ml), minocycline MICs (1 to 4 μg/ml), tigecycline MICs (1 to 4 μg/ml), azithromycin MICs (1 to 16 μg/ml), and clarithromycin MICs (0.125 to 8 μg/ml) being the lowest, on average. Synergistic effects of tetracyclines and macrolides were also observed.

KEYWORDS *Pythium insidiosum*, susceptibility profile, antibacterial agents

Human pythiosis is caused by an oomycete, *Pythium insidiosum*. Oomycota or oomycetes are a group of fungus-like stramenopiles. *P. insidiosum* is currently classified into four clades, clades I, II, III, and IV, based on the phylogenetic distribution of the internal transcribed spacer (ITS) region and cytochrome oxidase II (COX2) gene. Clade I isolates have been identified in the United States, and clade II isolates are mainly from Australia, India, Japan, New Zealand, Taiwan, and Thailand. Clade III isolates are from the United States, and clade IV comprises isolates from the Asia region and the Middle East (1, 2). This oomycete naturally inhabits soil, swampy areas, and stagnant freshwater. Accordingly, most observed opportunistic infections in Thailand tend to occur during the rainy seasons (3, 4). Human pythiosis has four clinical manifestations: vascular, ocular, skin and soft tissue, and disseminated infections (5). Vascular pythiosis is the most common form in Thailand, followed by ocular pythiosis. These infections result in devastating outcomes, with mortality rates being 10 to 40% in individuals with vascular pythiosis and an eye loss rate of 50% in those with ocular pythiosis (3, 4, 6).

Combination therapy, including radical surgery, itraconazole, terbinafine, and immunotherapy, had been the mainstay of treatment, according to the King Chulalongkorn Memorial Hospital (KCMH) research protocols, until December 2018. Itraconazole and terbinafine were used on the basis of their synergistic effects against Brazilian

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TABLE 1 *In vitro* susceptibility of Thai human *P. insidiosum* isolates to eight classes of antibacterial antibiotics[a]

		Clade II (n = 17)											Clade IV (n = 10)									
		No. of isolates with the following MIC (μg/ml):										GM MIC (μg/ml)	No. of isolates with the following MIC (μg/ml):									GM MIC (μg/ml)
Antibiotic class	Agent	0.125	0.25	0.50	1	2	4	8	16	32	>32		0.125	0.25	0.50	1	2	4	8	16	32	>32	
Tetracyclines	Doxycycline				1	4	9	2	1			3.69					2	5	3				4.29
	Minocycline				6	10	1					1.63				2	7	1					1.87
	Tigecycline				7	9	1					1.57				8	2						1.15
Macrolides	Azithromycin				1	7	9					3.13					2	3	4	1			5.28
	Clarithromycin	1		1	5	6	2	1				1.33				4	4	2					1.74
Beta-lactams	Cefazolin										17	>32										10	>32
	Ceftriaxone										17	>32										10	>32
	Ceftazidime										17	>32										10	>32
	Meropenem									1	16	32.00									1	9	32.00
Oxazolidinone	Linezolid						4	9	3	1		8.33						1	6	3			9.19
Glycopeptide	Vancomycin										17	>32										10	>32
Aminoglycosides	Amikacin										17	>32										10	>32
	Gentamicin										17	>32										10	>32
	Neomycin									1	16	32.00										10	>32
	Streptomycin								1	1	15	22.63										10	>32
	Tobramycin										17	>32										10	>32
Quinolones	Ciprofloxacin										17	>32										10	>32
	Levofloxacin										17	>32										10	>32
	Moxifloxacin								2	1	14	20.16								1	1	8	22.63
Polymyxins	Colistin (polymyxin E)								1	2	14	25.40									1	9	32.00
	Polymyxin B										17	>32										10	>32

[a]The MIC of each agent was determined by 100% inhibition of the mycelium by visual observation compared to the inhibition in the control well (no antibiotics). GM, geometric mean.

animal *P. insidiosum* isolates and a single case study of a patient with deep facial tissue infection who was effectively treated (7–9). However, synergistic effects of itraconazole and terbinafine were not observed against Thai clinical *P. insidiosum* isolates (3, 4, 6).

Recently, data on the *in vitro* and *in vivo* susceptibilities of Brazilian *P. insidiosum* isolates to antibacterial agents have shown that the MICs are more favorable than those of antifungal agents (7, 10–14). A prospective study from India likewise demonstrated the efficacy of topical linezolid, topical azithromycin, and oral azithromycin in treating *Pythium* keratitis. In that study, none of the patients required evisceration or enucleation; 33% were successfully treated with medications alone (15). In contrast, the most recent study of ocular pythiosis from Thailand using oral itraconazole and terbinafine in conjunction with various topical antifungal agents demonstrated an eye loss rate of 50%, and all patients required ocular surgery (3).

Antibacterial agents were first used in Thailand in 2019 as adjunctive therapy in two relapsed vascular pythiosis patients after itraconazole and immunotherapy failed (16). This study was conducted to better understand the susceptibility patterns of antibacterial classes against Thai human, Thai environmental, and non-Thai animal *P. insidiosum* isolates in different clades.

RESULTS

Tetracyclines (doxycycline [DOX], minocycline [MIN], tigecycline [TIG]), macrolides (azithromycin [AZM], clarithromycin [CLR]), and oxazolidinones (linezolid) had the lowest MIC values compared to those of the other antibacterial classes for all clades of Thai human, Thai environmental, and non-Thai animal *P. insidiosum* isolates (Tables 1 to 3). The geometric mean (GM) MICs of MIN, TIG, and CLR ranged from 1.00 to 2.18 μg/ml, followed by those for DOX and AZM, which had GM MICs of 2.72 to 5.28 μg/ml, and linezolid, which had GM MICs of 5.44 to 9.51 μg/ml. The other antibiotic agents had high GM MIC values (range, 10.77 to >32 μg/ml).

MIN, TIG, and CLR showed the lowest MIC values in this study. Among Thai human *P. insidiosum* isolates, MIN MICs ranged from 1 to 4 μg/ml (GM, 1.63 μg/ml) for clade II isolates and 1 to 4 μg/ml (GM, 1.87 μg/ml) for clade IV isolates; TIG MICs ranged from 1 to 4 μg/ml (GM, 1.57 μg/ml) for clade II isolates and 1 to 2 μg/ml (GM, 1.15 μg/ml) for clade IV isolates; CLR MICs ranged from 0.125 to 8 μg/ml (GM, 1.33 μg/ml) for clade II isolates and 1 to 4 μg/ml (GM, 1.74 μg/ml) for clade IV isolates. Among Thai

TABLE 2 *In vitro* susceptibility of Thai environmental *P. insidiosum* isolates against eight classes of antibacterial antibiotics[a]

		Clade II ($n = 4$)											Clade IV ($n = 8$)									
		No. of isolates with the following MIC (μg/ml):										GM MIC	No. of isolates with the following MIC (μg/ml):									GM MIC
Antibiotic class	Agent	0.125	0.25	0.50	1	2	4	8	16	32	>32	(μg/ml)	0.125	0.25	0.50	1	2	4	8	16	32	>32	(μg/ml)
Tetracyclines	Doxycycline						3	1				4.76					3	3	1	1			4.00
	Minocycline					4						2.00					7	1					2.18
	Tigecycline					4						2.00					7	1					2.18
Macrolides	Azithromycin					1	2		1			4.76					2	4	2				4.00
	Clarithromycin					4						2.00				2	5	1					1.83
Beta-lactams	Cefazolin										4	>32										8	>32
	Ceftriaxone										4	>32										8	>32
	Ceftazidime										4	>32										8	>32
	Meropenem									1	3	32.00										8	>32
Oxazolidinone	Linezolid						1	1	2			9.51							6	2			9.51
Glycopeptide	Vancomycin										4	>32										8	>32
Aminoglycosides	Amikacin										4	>32										8	>32
	Gentamicin										4	>32										8	>32
	Neomycin										4	>32										8	>32
	Streptomycin										4	>32										8	>32
	Tobramycin										4	>32										8	>32
Quinolones	Ciprofloxacin										4	>32										8	>32
	Levofloxacin										4	>32										8	>32
	Moxifloxacin								1	1	2	22.63										8	>32
Polymyxins	Colistin (polymyxin E)								1	1	2	22.63								1		7	16.00
	Polymyxin B										4	>32										8	>32

[a]The MIC of each agent was determined by 100% inhibition of the mycelium by visual observation compared to the inhibition in the control well (no antibiotics). GM, geometric mean.

environmental isolates, all clade II isolates had MIN, TIG, and CLR MICs of 2 μg/ml (GM, 2.00 μg/ml); *P. insidiosum* clade IV isolates had MIN, TIG, and CLR MICs of 2 to 4 μg/ml (GM, 2.18 μg/ml), 2 to 4 μg/ml (GM, 2.18 μg/ml), and 1 to 4 μg/ml (GM, 1.83 μg/ml), respectively. Among clade I and clade II non-Thai animal *P. insidiosum* isolates, MIN MICs were 0.25 to 4 μg/ml (GM, 1.08 μg/ml) for clade I isolates and 2 μg/ml (GM, 2.00 μg/ml) for clade II isolates; TIG MICs were 0.5 to 2 μg/ml (GM, 1.08 μg/ml) for clade I isolates and 2 μg/ml (GM, 2.00 μg/ml) for clade II isolates; CLR MICs ranged from 0.125 to 2 μg/ml (GM, 1.00 μg/ml) for clade I isolates and 1 to 2 μg/ml (GM, 1.41 μg/ml) for clade II isolates.

DOX and AZM had the second lowest MIC values. Among Thai human *P. insidiosum*

TABLE 3 *In vitro* susceptibility of non-Thai animal *P. insidiosum* isolates against eight classes of antibacterial antibiotics[a]

		Clade I ($n = 9$)											Clade II ($n = 2$)									
		No. of isolates with the following MIC (μg/ml):										GM MIC	No. of isolates with the following MIC (μg/ml):									GM MIC
Antibiotic class	Agent	0.125	0.25	0.5	1	2	4	8	16	32	>32	(μg/ml)	0.125	0.25	0.5	1	2	4	8	16	32	>32	(μg/ml)
Tetracyclines	Doxycycline				1	2	5		1			3.43						2					4.00
	Minocycline		1	1	5		2					1.08					2						2.00
	Tigecycline			1	6	2						1.08					2						2.00
Macrolides	Azithromycin					6	2	1				2.72					1	1					2.83
	Clarithromycin	1			5	3						1.00				1	1						1.41
Beta-lactams	Cefazolin								1		8	>32										2	>32
	Ceftriaxone										9	>32										2	>32
	Ceftazidime										9	>32										2	>32
	Meropenem									5	4	32.00										2	>32
Oxazolidinone	Linezolid						5	4				5.44							2				8.00
Glycopeptide	Vancomycin										9	>32										2	>32
Aminoglycosides	Amikacin										9	>32										2	>32
	Gentamicin								1	3	5	26.91										2	>32
	Neomycin									2	7	32.00										2	>32
	Streptomycin								1	3	5	26.91										2	>32
	Tobramycin										9	>32										2	>32
Quinolones	Ciprofloxacin									1	8	32.00										2	>32
	Levofloxacin										9	>32										2	>32
	Moxifloxacin							1	2	3	3	20.16										2	>32
Polymyxins	Colistin (polymyxin E)							4	3		2	10.77								1		1	16.00
	Polymyxin B										9	>32										2	>32

[a]The MIC of each agent was determined by 100% inhibition of the mycelium by visual observation compared to the inhibition in the control well (no antibiotics). GM, geometric mean.

TABLE 4 *In vitro* activity of combinations of azithromycin, clarithromycin, minocycline, doxycycline, and tigecycline against Thai human *P. insidiosum* isolates[a]

		Clade II ($n = 17$)		Clade IV ($n = 10$)	
Drug combination	Interpretation of activity	No. of isolates	FICI range (GM)	No. of isolates	FICI range (GM)
AZM-MIN	Synergism	13	0.19–0.56 (0.31)	9	0.09–0.38 (0.23)
	Indifference	4	0.56–0.75 (0.60)	1	0.75 (0.75)
AZM-TIG	Synergism	14	0.09–0.38 (0.24)	10	0.25–0.38 (0.31)
	Indifference	3	0.56–0.63 (0.59)		
CLR-MIN	Synergism	17	0.09–0.50 (0.30)	10	0.09–0.31 (0.15)
	Indifference				
CLR-TIG	Synergism	17	0.15–0.38 (0.24)	10	0.19–0.49 (0.31)
	Indifference				
MIN-TIG	Synergism	17	0.19–0.38 (0.28)	9	0.19–0.38 (0.28)
	Indifference			1	0.63 (0.63)
DOX-AZM	Synergism	17	0.19–0.42 (0.27)	8	0.19–0.50 (0.31)
	Indifference			2	0.75 (0.75)
DOX-CLR	Synergism	17	0.25–0.50 (0.28)	9	0.25–0.50 (0.35)
	Indifference			1	0.56 (0.56)
DOX-TIG	Synergism	17	0.19–0.50 (0.32)	10	0.18–0.50 (0.28)
	Indifference				

[a]AZM, azithromycin; CLR, clarithromycin; MIN, minocycline; TIG, tigecycline; DOX, doxycycline; FICI, fractional inhibitory concentration index. Interpretations of activity were as follows: antagonism, FICI > 4; indifference, $0.5 <$ FICI < 4; synergism, FICI ≤ 0.5.

isolates, DOX MICs ranged from 1 to 16 μg/ml (GM, 3.69 μg/ml) for clade II isolates and 2 to 8 μg/ml (GM, 4.29 μg/ml) for clade IV isolates; AZM MICs ranged from 1 to 4 μg/ml (GM, 3.13 μg/ml) for clade II isolates and 2 to 16 μg/ml (GM, 5.28 μg/ml) for clade IV isolates. Among Thai environmental isolates, DOX MICs ranged from 4 to 8 μg/ml (GM, 4.76 μg/ml) for clade II isolates and 2 to 16 μg/ml (GM, 4.00 μg/ml) for clade IV isolates; AZM MICs ranged from 2 to 16 μg/ml (GM, 4.76 μg/ml) for clade II isolates and 2 to 8 μg/ml (GM, 4.00 μg/ml) for clade IV isolates. For the other group, non-Thai animal *P. insidiosum* isolates, DOX MICs were 1 to 16 μg/ml (GM, 3.43 μg/ml) for clade I isolates and 4 μg/ml (GM, 4.00 μg/ml) for clade II isolates; AZM MICs ranged from 2 to 8 μg/ml (GM, 2.72 μg/ml) for clade I isolates and 2 to 4 μg/ml (GM, 2.83 μg/ml) for clade II isolates.

Linezolid MICs ranged from 4 to 32 μg/ml (GM, 8.33 μg/ml) for the clade II isolates and 4 to 16 μg/ml (GM, 9.19 μg/ml) for the clade IV isolates among Thai human *P. insidiosum* isolates and from 4 to 16 μg/ml (GM, 9.51 μg/ml) for the clade II isolates and 8 to 16 μg/ml (GM, 9.51 μg/ml) for the clade IV isolates among Thai environmental *P. insidiosum* isolates. However, among non-Thai animal *P. insidiosum* isolates, linezolid MICs were 4 to 8 μg/ml (GM, 5.44 μg/ml) for clade I isolates and 8 μg/ml (GM, 8.00 μg/ml) for clade II isolates. Mycelium growth of all isolates was not inhibited by beta-lactams, glycopeptides, aminoglycosides, quinolones, or polymyxins (Tables 1 to 3).

The *in vitro* activities of the tetracyclines combined with the macrolides are displayed in Tables 4 to 6. Synergistic effects were observed in more than 90% of the Thai human *P. insidiosum* isolates (94.85% of clade II isolates and 93.75% of clade IV isolates) and Thai environmental *P. insidiosum* isolates (90.63% of clade II isolates and 90.63% of clade IV isolates) and more than 85% of non-Thai animal *P. insidiosum* isolates (97.22% of clade I isolates and 87.50% of clade II isolates). Antagonistic interactions were not observed in this study.

DISCUSSION

This is the largest *in vitro* susceptibility study of human, environmental, and animal *P. insidiosum* isolates to date examining their antibacterial susceptibility patterns and evaluating new treatment options for the devastating disease caused by this organism. This study reveals that tetracyclines and macrolides have MICs 10 to 100 times lower than those of antifungal agents determined in previous studies (3, 6). These findings correlate with published data from Brazil (10, 11, 13). More importantly, synergistic

TABLE 5 *In vitro* activity of combinations of azithromycin, clarithromycin, minocycline, doxycycline, and tigecycline against Thai environmental *P. insidiosum* isolates[a]

		Clade II ($n = 4$)		Clade IV ($n = 8$)	
Drug combination	Interpretation of activity	No. of isolates	FICI range (GM)	No. of isolates	FICI range (GM)
AZM-MIN	Synergism	3	0.08–0.50 (0.23)	6	0.12–0.25 (0.21)
	Indifference	1	0.75 (0.75)	2	0.63–0.75 (0.68)
AZM-TIG	Synergism	3	0.19–0.31 (0.24)	7	0.19–0.38 (0.28)
	Indifference	1	0.63 (0.63)	1	0.56 (0.56)
CLR-MIN	Synergism	4	0.08–0.31 (0.17)	8	0.09–0.38 (0.19)
	Indifference				
CLR-TIG	Synergism	4	0.19–0.37 (0.26)	8	0.16–0.38 (0.25)
	Indifference				
MIN-TIG	Synergism	3	0.25 (0.25)	8	0.18–0.38 (0.23)
	Indifference	1	0.63 (0.63)		
DOX-AZM	Synergism	4	0.25–0.38 (0.31)	7	0.25–0.38 (0.34)
	Indifference			1	0.56 (0.56)
DOX-CLR	Synergism	4	0.19–0.31 (0.26)	6	0.25–0.38 (0.30)
	Indifference			2	0.56–0.75 (0.65)
DOX-TIG	Synergism	4	0.19–0.31 (0.25)	8	0.18–0.31 (0.25)
	Indifference				

[a]AZM, azithromycin; CLR, clarithromycin; MIN, minocycline; TIG, tigecycline; DOX, doxycycline; FICI, fractional inhibitory concentration index. Interpretations of activity were as follows: antagonism, FICI > 4; indifference, $0.5 <$ FICI < 4; synergism, FICI ≤ 0.5.

effects of antimicrobial agents against Thai *P. insidiosum* isolates were observed, highlighting new potential treatment options for human pythiosis in Thailand.

Until recently in Thailand, itraconazole and terbinafine were used to treat all pythiosis patients, despite their unfavorable MICs and lack of synergy (4). Pragmatically, it has been very challenging to maintain therapeutic serum itraconazole levels throughout 6 to 12 months of treatment (recognizing that there are no standard guidelines for ideal therapeutic drug levels or an MIC breakpoint interpretation for *P. insidiosum*). Approximately 25% of patients cannot receive terbinafine because it is unavailable in rural Thailand (6). However, these patients can generally receive azithromycin, clarithromycin, and doxycycline without geographic limitations or restrictions from health insurance. With the significantly lower MICs of tetracycline and macrolide classes, there is a reasonable probability that patients' blood or tissue drug concentrations would be above the MICs. For instance, oral doxycycline at 200 mg and clarithromycin at 500 mg can result in serum drug concentrations of 2 to 4 μg/ml and 2 to 3 μg/ml, respectively (11). In fact, treatment by the adjunctive use of antibacterial agents (azithromycin-

TABLE 6 *In vitro* activity of combinations of azithromycin, clarithromycin, minocycline, doxycycline, and tigecycline against non-Thai animal *P. insidiosum* isolates[a]

		Clade I ($n = 9$)		Clade II ($n = 2$)	
Drug combination	Interpretation	No. of isolates	FICI range (GM)	No. of isolates	FICI range (GM)
AZM-MIN	Synergism	8	0.14–0.38 (0.22)	2	0.19–0.29 (0.23)
	Indifference	1	1.13 (1.13)		
AZM-TIG	Synergism	8	0.25–0.38 (0.33)	1	0.38 (0.38)
	Indifference	1	0.75 (0.75)	1	0.67 (0.67)
CLR-MIN	Synergism	9	0.27–0.38 (0.32)	2	0.31–0.38 (0.34)
	Indifference				
CLR-TIG	Synergism	9	0.27–0.49 (0.35)	2	0.37 (0.37)
	Indifference				
MIN/TIG	Synergism	9	0.25–0.49 (0.31)	2	0.28–0.31 (0.29)
	Indifference				
DOX-AZM	Synergism	9	0.28–0.50 (0.34)	1	0.29 (0.29)
	Indifference			1	0.63 (0.63)
DOX-CLR	Synergism	9	0.19-038 (0.33)	2	0.50 (0.50)
	Indifference				
DOX-TIG	Synergism	9	0.25–0.50 (0.42)	2	0.37–0.38 (0.37)
	Indifference				

[a]AZM, azithromycin; CLR, clarithromycin; MIN, minocycline; TIG, tigecycline; DOX, doxycycline; FICI, fractional inhibitory concentration index. Interpretations of activity were as follows: antagonism, FICI > 4; indifference, $0.5 <$ FICI < 4; synergism, FICI ≤ 0.5.

TABLE 7 Characteristics of the 50 isolates of *Pythium insidiosum* used in this study

Isolate no.	ITS GenBank accession no. or CBS no.[a]	Isolate source	Country	Clade
1.	KX389263	Human, cerebral	Thailand	II
2.	CBS 119454	Human, cerebral	Thailand	IV
3.	AY151173	Human, vascular	Thailand	II
4.	GU137329	Human, vascular	Thailand	II
5.	FJ917395	Human, vascular	Thailand	II
6.	JQ409330	Human, vascular	Thailand	II
7.	KX371893	Human, vascular	Thailand	II
8.	KX371894	Human, vascular	Thailand	II
9.	KX371895	Human, vascular	Thailand	II
10.	FJ917393	Human, vascular	Thailand	II
11.	GQ260121	Human, vascular	Thailand	II
12.	GQ260123	Human, vascular	Thailand	II
13.	CBS 119452	Human, vascular	Thailand	II
14.	CBS 119453	Human, vascular	Thailand	II
15.	GU812440	Human, vascular	Thailand	IV
16.	GQ260120	Human, vascular	Thailand	IV
17.	GQ260122	Human, vascular	Thailand	IV
18.	FJ917390	Human, vascular	Thailand	IV
19.	CBS 673.85	Human, vascular	Thailand	IV
20.	JQ409332	Human, ocular	Thailand	II
21.	GQ260119	Human, ocular	Thailand	II
22.	GQ260118	Human, ocular	Thailand	II
23.	CBS 119455	Human, ocular	Thailand	II
24.	FJ917389	Human, ocular	Thailand	IV
25.	GQ475491	Human, ocular	Thailand	IV
26.	GQ260125	Human, ocular	Thailand	IV
27.	GQ260124	Human, ocular	Thailand	IV
28.	EF016908	Environment, rice irrigation in Chiang Rai	Thailand	II
29.	EF016902	Environment, reservoir in Chiang Rai	Thailand	II
30.	EF016910	Environment, reservoir in Chiang Rai	Thailand	II
31.	EF016885	Environment, reservoir in Lumpang	Thailand	II
32.	EF016883	Environment, reservoir in Lumphun	Thailand	IV
33.	EF016870	Environment, reservoir in Lumphun	Thailand	IV
34.	EF016866	Environment, irrigation channel in Lumphun	Thailand	IV
35.	EF016878	Environment, reservoir in Lumphun	Thailand	IV
36.	EF016895	Environment, reservoir in Lumpang	Thailand	IV
37.	EF016879	Environment, reservoir in Lumphun	Thailand	IV
38.	FJ917392	Environment, reservoir in Lopburi	Thailand	IV
39.	EF016875	Environment, reservoir in Lumphun	Thailand	IV
40.	CBS 573.85	Equine	Costa Rica, USA	I
41.	CBS 574.85	Equine	Costa Rica, USA	I
42.	CBS 575.85	Equine	Costa Rica, USA	I
43.	CBS 576.85	Equine	Costa Rica, USA	I
44.	CBS 577.85	Equine	Costa Rica, USA	I
45.	CBS 578.85	Equine	Costa Rica, USA	I
46.	CBS 579.85	Equine	Costa Rica, USA	I
47.	CBS 580.85	Equine	Costa Rica, USA	I
48.	CBS 10155	Equine	Brazil	I
49.	CBS 702.83	Equine	Japan	II
50.	CBS 777.84	Mosquito larva	India	II

[a]ITS, internal transcribed spacer; CBS, Centraalbureau voor Schimmelcultures.

doxycycline and clarithromycin-doxycycline), guided by *in vitro* susceptibility results, was successful in two patients in Thailand with relapses of vascular pythiosis (16).

The mechanisms of action of tetracyclines and macrolides against *P. insidiosum* remain unclear. However, it has been hypothesized that the potential mechanisms could include inhibition of protein synthesis, cell wall synthesis, and/or amino acid transport (11). We observed that clade I of non-Thai animal *P. insidiosum* isolates tended to have the lowest MICs. Broader MIC ranges of clarithromycin were found for clade II Thai human isolates and clade I non-Thai animal isolates. These findings imply that clinical *in vitro* susceptibility testing should be performed, if feasible, to determine individual treatment options. The necessity of susceptibility testing is also supported by

the findings of McMeekin et al., who studied the growth of a Thai human *P. insidiosum* isolate and found that it was stimulated by streptomycin, while the growth of other isolates was inhibited by the same concentration of streptomycin (17). This result raises the concern that some antibiotics might have unpredictable effects on the inhibition of *P. insidiosum*.

Limitations of this study include the limited number of isolates tested and the underrepresentation of all clades among all isolate sources. There were no Thai human or environmental clade I or clade III isolates in this study, although this likely represents an accurate sampling, as *P. insidiosum* clade I isolates have not been reported in Thai patients or the Thai environmental *P. insidiosum* clade III isolates are also not commonly found in the Thai environment (1, 2). Among non-Thai animal *P. insidiosum* isolates, clade II isolates were not examined in this study due to its commercial unavailability during the study period.

With those limitations, we are still hopeful that tetracyclines (doxycycline, minocycline, and tigecycline) and macrolides (azithromycin and clarithromycin) may offer new treatment options for human pythiosis in Thailand. Further clinical trials are needed to evaluate the clinical efficacy of these and other antibacterial agents.

MATERIALS AND METHODS

The study was approved by the Chulalongkorn University Institutional Review Board (IRB) (certificate of authenticity; COA no. 099/2019, IRB no. 760/61).

A total of 50 *P. insidiosum* isolates were tested, including Thai human ($n = 27$), Thai environmental ($n = 12$), and non-Thai animal ($n = 11$) *P. insidiosum* isolates. The Thai human isolates were derived from patients treated according to the KCMH research protocol (July 2002 to June 2019) ($n = 22$), and another 5 isolates were received from the Dutch Centraalbureau voor Schimmelcultures (CBS Bank) (Table 7).

Among the 27 Thai human *P. insidiosum* isolates, 2 were from cerebral lesions, 17 were from arterial clots, and 8 were from corneas. Among those 27 Thai human *P. insidiosum* isolates, 17 were clade II and 10 were clade IV. *P. insidiosum* environmental isolates were obtained from a water reservoir ($n = 1$) and from the Faculty of Medicine, Chiang Mai University ($n = 11$). Among the 12 Thai environmental *P. insidiosum* isolates, 4 were in clade II and 8 were in clade IV. Of the animal isolates from other countries, 8 were equine isolates from the United States, 1 was an equine isolate from Brazil, 1 was an equine isolate from Japan, and 1 was a mosquito larva isolate from India. Among the 11 non-Thai animal *P. insidiosum* isolates, both clades I ($n = 9$) and II ($n = 2$) were represented (Table 7). The genus and species of the Thai *P. insidiosum* isolates were confirmed using a PCR-based assay of the ITS region and/or COX2 gene, whereas the clades were classified according to DNA sequencing of the ITS region (18–20).

Broth microdilution was performed according to Clinical and Laboratory Standards Institute (CLSI) document M38-A2 guidelines for filamentous fungi (21), modified for *P. insidiosum* against eight classes of antibacterial antibiotics (Tables 1 to 3). All standard antimicrobial powders were commercially obtained from Sigma-Aldrich (St. Louis, MO, USA). Briefly, zoospores were obtained by zoosporogenesis induction (22). The inoculum (2×10^3 to 3×10^3 zoospores/ml) was counted by use of a Neubauer chamber and diluted in RPMI 1640 broth, pH 7.0 (with glucose and L-glutamine). The agents were prepared by 2-fold dilution in RPMI 1640 over a concentration range of 0.125 to 32 μg/ml. All assays were performed in triplicate. The MICs of each agent were determined by 100% inhibition of mycelium growth after 24 and 48 h of incubation at 37°C by visual observation (10, 11, 13, 14). The *in vitro* synergy of the tetracyclines and macrolides was determined according to the checkerboard technique (16).

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