An initial survey of 150 horses from Thailand for anti-*Pythium insidiosum* antibodies

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Abstract

Objectives: *Pythium insidiosum* causes a deadly condition, called pythiosis, in humans and other animals. The organism has been identified in tropical and subtropical environments worldwide. Since 1985, human pythiosis has been increasingly reported from Thailand. Seroprevalence studies estimated that ~32,000 Thai people have been exposed to the pathogen. In 2018, the first animal pythiosis case in Thailand was diagnosed in a horse. In this study, we surveyed anti-*P. insidiosum* antibodies in a sample of the Thai equine population.

Results: Serum samples were available from 150 out of 6,353 registered horses distributed across Thailand. ELISA detected the anti-*P. insidiosum* antibodies in three horses. Immunochromatography and Western blot confirmed the presence of the antibodies in one of the ELISA-positive horses. Based on one positive out of 150 tested, the *Pythium* seroprevalence in the Thai equine population was 0.7%, which is 10 times higher than that of the Thai human population. The seroprevalence of the anti-*P. insidiosum* antibodies in a small sample of horses suggests a higher incidence of *pythiosis* in horses than in humans. Larger studies will be necessary to obtain the full picture of the epidemiology of animal pythiosis in Thailand.

Introduction

Pythium insidiosum is capable of infecting humans, horses and dogs residing in tropical and subtropical areas across the globe [1, 2]. The pathogen has been identified in water and soil samples from around the world [3–7]. Direct contact with *P. insidiosum* can initiate the deadly condition called pythiosis [1, 2, 8–10]. Laboratory tests are required to make a definitive diagnosis of pythiosis [1, 10–12]. Early and proper treatment may substantially improve the clinical outcomes for pythiosis patients [1, 2, 10].

Data on the epidemiology of pythiosis is limited. Since the first case in 1985, human pythiosis has been increasingly reported from all over Thailand [2, 10, 13–19], where *P. insidiosum* is ubiquitous in the environment [3, 4]. A seroprevalence study estimated that ~32,000 Thai people have been exposed to the pathogen [20]. In the other endemic countries (i.e., Brazil, Costa Rica, the United States, and Australia), human pythiosis is relatively rare, whereas the disease in animals (i.e., horses and dogs) is much more prevalent [1, 21]. It is unknown if it is host, pathogen, or environmental factors that contribute to the different prevalence of human and animal pythiosis.

Animal pythiosis was reported in Thailand in a horse in 2018 [22] and a dog in 2020 [23]. The relative paucity of reports of animal pythiosis in Thailand may result from under-recognition and under-diagnosis of the disease. To acquire evidence on the exposure of animals to the pathogen, we surveyed serum anti-*P. insidiosum* antibodies in 150 horses distributed across Thailand, using 3 established serological tests: a protein A/G-based enzyme-linked immunosorbent assay (ELISA); a protein A/G-based immunochromatographic test (ICT); and Western blot analysis [11, 12, 20]. This study is a first step leading to a better understanding of the epidemiology of animal pythiosis in Thailand.

Materials And Methods

Serum samples from 150 healthy horses were made available from the Faculty of Veterinary Medicine, Kasetsart University, Thailand (Table 1). The horses resided in northern (n = 43), central (n = 86), eastern (n = 14), and southern (n = 7) regions of Thailand. Serum samples from a Thai horse with culture-proven pythiosis and a healthy blood donor served as the positive and negative controls, respectively. The serum sample were tested (in duplicate) to detect the anti-*P. insidiosum* antibodies using an established protein A/G-based ELISA [11] with some modifications. Briefly, a 96-well polystyrene plate (Corning) was coated with 100 µl of 5 µg/ml culture filtrate antigen (prepared from the human-isolated *P. insidiosum* strain Pi-S [24]) in 0.1 M carbonate buffer (pH 9.6) and 1.5% NaCl (4 °C overnight), washed 4 times with PBS and 0.05% tween-20 (PBS-T), blocked with 250 µl of 0.5% bovine serum albumin (Merck) in PBS (37 °C for 80 min), and washed 4 times again with PBS-T. A diluted serum sample (1:1,600 in PBS; 100 µl) was incubated in each well (37°C for 1 hr) and was washed 4 times with PBS-T. The peroxidase-conjugated protein A/G (Bio-Rad) (1:100,000 in PBS; 100 µl) was added to each well and incubated at 37°C for 1 hr. After a washing step as above, the chromogenic substrate (Tetramethylbenzidine and H₂O₂; 100 µl) (Thermo Scientific) was added into each well and incubated in the dark at room temperature for 3 min. The enzymatic reaction was stopped by applying 100 µl of 0.5 N H₂SO₄. The optical density (OD) of every serum sample was measured (at 450-nm) by an Infinite 200 Pro microplate reader (Tecan). The obtained OD minus the blank OD (PBS) was divided by that of the negative control to provide an ELISA value (EV). The ELISA⁺ cutoff point was determined to be the mean EV of all tested serum samples plus 3 SDs, as previously described by other investigators [12, 20, 25].

All ELISA-positive sera, together with an equivalent number of randomly-selected ELISA-negative samples, were tested for the presence of anti-*P. insidiosum* antibodies by ICT [12] and Western blot analysis [20]. The positive and negative control sera were tested in parallel. A protein A/G-based ICT strip was dipped in 100 ul of each serum sample, diluted 1:5,000 in 0.15 M PBS (pH 7.4), for 30 min. The appearance of both test and control lines indicated an ICT-positive result, while the presence of only the control line indicated an ICT-negative result. For Western blot analysis, CFA of *P. insidiosum* [24] was separated by SDS-PAGE (4% stacking and 12% resolving gels) using a Bio-Rad MiniProteon II apparatus (setting: 100v for 90 min) and blotted onto nitrocellulose membrane (Bio-Rad) using a Bio-Rad Mini Trans-Blot apparatus (setting: 100v for 60 min). The blotted membrane was blocked with 5% skimmed milk in PBS with 0.1% Tween-20 (PBS-T) at room temperature for 60 min, and incubated with a serum sample, diluted 1:2,000 in 1% skimmed milk in PBS-T, at room temperature for 3 hr. After a washing step, the A/G protein, conjugated with horseradish peroxidase, was added to the membrane and incubated at room temperature for 2 hr. Western blot signals were generated using 0.03% diaminobenzidine tetrahydrochloride, 0.05% cobalt chloride, and 0.06% hydrogen peroxide in PBS.

The provinces where the equine serum samples were obtained were plotted on an on-line Thailand map using the Microreact software [26]. The most recent numbers of registered horses and farms were derived from the Department of Livestock Development, Ministry of Agriculture and Cooperatives, Thailand

(<http://en.dld.go.th/>). Means, standard deviations (SD), and distribution histogram of EVs from all equine serum samples were obtained by using the EXCEL program (version 16.35).

Results

Serum samples were obtained from 150 horses (on 27 farms) distributed in 8 provinces across Thailand (*Table 1*). The geographic locations of these provinces (i.e., Chiang Rai, Payao, Suphanburi, Kanchanaburi, Ratchaburi, Chachoengsao, Chonburi, and Trang) is plotted on a map (*Figure 1*), which can be accessed online at <https://microreact.org/project/f2QcmQktR>. All serum samples (identifier [IDs]: #1–150) were screened for anti-*P. insidiosum* antibodies by protein A/G-based ELISA [11]. With the “mean+3SDs” ELISA cut-off point (EV = 40.7), sera from 3 horses in Suphanburi (ID #10; EV = 47.0), Kanchanaburi (ID #98; EV = 60.9), and Trang (ID #132; EV = 86.1) provinces tested positive, while that from the other 147 horses (average EV = 8.2; range: 1.3–33.5) were negative (*Figure 2A*).

Three ELISA-positive sera (IDs #10, 98, and 132), 3 randomly-selected ELISA-negative sera (IDs #4, 19, and 77), and the positive and negative control sera were further analyzed using ICT and Western blot. The ICT test confirmed antibodies in the positive control and in one of the ELISA-positive samples (ID #132; *Figure 2B*). Likewise, for the Western blot analyses, only the serum ID #132 sample showed 7 prominent immunoreactive bands (i.e., 12, 18, 22, 27, 40, 55, 120 kDa) comparable with the reference bands of the positive control serum (from a culture-proven horse with pythiosis) (*Figure 2C*). In contrast, the other 2 ELISA-positive sera, which had lower values in the ELISA test (IDs #10 and 98), the ELISA-negative sera (IDs #4, 19, and 77), and the negative control serum all resulted in only a few faint immunoreactive bands (i.e., 32, 40, and 120 kDa) (*Figure 2C*).

The presence of anti-*P. insidiosum* antibodies was detected in one out of 150 horses (0.7%) by all 3 serological tests (i.e., ELISA, ICT, and Western blot). A total of 6,353 horses were registered in Thailand, according to the 2014 report of the Department of Livestock Development, Thai Ministry of Agriculture and Cooperatives (<http://en.dld.go.th/>). Based on our sample, the number of horses with a detectable level of anti-*P. insidiosum* antibodies is estimated to be 6.7 in 1,000 or 42 in the registered equine population in Thailand.

Discussion

Two seroprevalence studies of anti-*P. insidiosum* antibodies were independently conducted by Weiblen et al. in Brazil [25] and Lohnoo et al. in Thailand [20]. Weiblen et al. tested the sera from 1,002 out of ~550,000 horses in the Rio Grande do Sul State of Brazil and reported the seroprevalence of 11.1% [25, 27]. Lohnoo et al. assessed the sera from 2,641 people and estimated the seroprevalence of 0.07% (or 32,000 in the entire Thai human population) [20]. In the current study, serum samples were obtained from 150 out of 6,353 horses registered in Thailand (*Table 1, Figure 1*). Based on the consensus results from 3 established serological tests (*Figure 2*), the seroprevalence of anti-*P. insidiosum* antibodies in 150 horses was 0.7% (or an estimated 42 in the entire Thai equine population). Such seroprevalence in Thai horses

(0.7%) is 10 times higher than that estimated for Thai people (0.07%) [20] and 17 times lower than that of Brazilian horses (11.1%) [25]. A higher seroprevalence implies a higher exposure rate of a target population to *P. insidiosum*.

Lower seroprevalence (0.7 vs. 11.1%) and fewer horses than in the Rio Grande do Sul State of Brazil (6,353 vs. 550,000) [25, 27] explain why the number of horses with pythiosis in Thailand is minimal with just one case reported to date [28] vs. hundreds of Brazilian horses reported with the disease [29–36]. The striking difference in seroprevalences of the Thai (0.7%) and Brazilian (11.1%) horses could imply that Thai people, by comparison, might have a lower chance of having environmental exposure to *P. insidiosum*. However, the reported cases of human pythiosis from Thailand are much higher than those from Brazil [10, 13, 13, 16, 16, 37–39]. A host or pathogen factors might contribute to such a different prevalence of human pythiosis: Thalassemia is a common underlying condition in Thai patients with pythiosis [2, 40]; Genotypes of the Thai *P. insidiosum* strains are different from that of the Brazilian strains [41, 42].

Conclusion

We report an initial survey of anti-*P. insidiosum* antibodies in 150 horses from Thailand, using 3 established serological methods (i.e., ELISA, ICT, and Western blot). The seroprevalence of the antibodies in the Thai equine population (0.7%) was markedly higher than that of the Thai human population (0.07%), but much lower than that of the Brazilian equine population (11.1%). The antibody surveillances reported by our group and other investigators were undertaken to promote a better understanding of the epidemiology and host susceptibility of pythiosis in different geographic locations. Larger studies will be necessary to obtain a complete picture of the epidemiology of pythiosis.

Limitations

Serum samples, used in this study, were derived from a limited number of horses (i.e., 150 out of 6,353 horses). Besides, the recruited horses were disproportionally distributed throughout Thailand. Therefore, the resulting seroprevalence is statistically limited.

Abbreviations

CFA	Culture filtrate antigen
ELISA	Enzyme-linked immunosorbent assay
EV	ELISA value
ICT	Immunochromatographic test
ID	Identifier

OD	Optical density
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline and 0.05% tween-20
SD	Standard deviation

Declarations

Ethics approval and consent to participate

This study and the use of serum samples were approved by the Institutional Animal Care and Use Committee, Kasetsart University, Bangkok, Thailand (assigned number: ACKU61-VET-093), and the Committee for Research, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand (assigned number: MURA2020/547).

Availability of data and material

The *P. insidiosum* strain Pi-S used for crude protein preparation is available upon request.

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Consent for publication

Not applicable.

Competing interests

None.

Author's contributions

Study design (ZMH, AL, PC, TK); Experiment & Methodology (ZMH, AL, WP, CY, TL, WY, YK, PP, PS, TR, CJ, TK); Data collection (ZMH, AL, WP, CY, TL, TR, CJ, TK); Data analysis (ZMH, AL, WP, CY, PC, TK);

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Table

Table 1. Geographic locations, number of recruited horses, and serological test results of 150 equine serum samples.

Region of Thailand	Province (reference code)	GPS coordination ^a		Tested horses	Horse farms	Positive test results		
		Latitude	Longitude			ELISA ^b	ICT ^c	WB ^d
Northern	Chiang Rai (A)	19.9105	99.8406	29	2	-	-	-
	Payao (B)	19.2154	100.2024	14	2	-	-	-
Central	Suphanburi (C)	14.4745	100.1177	10	6	1	-	-
	Kanchanaburi (D)	14.1011	99.4179	38	5	1	-	-
	Ratchaburi (E)	13.5283	99.8134	38	9	-	-	-
Eastern	Chachoengsao (F)	13.6904	101.0780	8	1	-	-	-
	Chonburi (G)	13.3611	100.9847	6	1	-	-	-
Southern	Trang (H)	7.5645	99.6239	7	1	1	1	1
Total				150	27	3	1	1

Footnote:

^a Global Positioning System coordination of the provinces where the horse sera were obtained

^b Protein A/G-based enzyme linked immunosorbent assay

^c Protein A/G-based immunochromatographic test

^d Western blot analysis

Figures

Figure 1

The map indicates the geographic locations of 8 provinces in the Northern [Chiang Rai (A) and Payao (B)], Central [Suphanburi (C), Kanchanaburi (D) and Ratchaburi (E)], Eastern [Chachoengsao (F) and Chonburi (G)], and Southern [Trang (H)] regions of Thailand, where the serum samples of 150 horses were obtained. The map was created by the Microreact program (the online version: <https://microreact.org/project/f2QcmQktR>). A green dot represents the province with ELISA-negative sera.

A yellow dot shows the province with a seropositive sample by only ELISA. A red dot reveals the province with a seropositive sample by all 3 serological tests (ELISA, ICT, and Western blot). The numbers (#10, 98, and 132) indicate IDs of the seropositive samples. (Abbreviations: ELISA, enzyme-linked immunosorbent assay; ICT, immunochromatographic test)

Figure 2

Serological test results of the serum samples from 150 horses in Thailand: (A) Histogram shows the distribution of ELISA values of all samples according to frequencies and ranges (i.e., 0.0-2.5, 2.6-5.0, 5.1-7.5, 7.6-10.0, ...). The cut-off point is derived from the mean ELISA value plus 3 standard deviations of all

samples. The numbers represent ELISA-positive samples (IDs 10, 98, and 132) and randomly-selected ELISA-negative samples (IDs 4, 19, and 77) for ICT and Western blot analysis. (B) ICT results of the ELISA-positive and ELISA-negative samples. Arrows indicate the test line (T). C is the control line. (C) Western blot analysis of the ELISA-positive and ELISA-negative samples. M represents the molecular weight markers (kDa). Arrowheads indicate major immunoreactive bands. (Abbreviations: ELISA, enzyme-linked immunosorbent assay; ICT, immunochromatographic test; PC, positive control serum; NC, negative control serum)