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Blastocystis subtype 5: Predominant subtype on pig farms, Thailand

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ABSTRACT

Blastocystis is a unicellular protist most commonly detected in humans and a variety of animals. The predominant mode of its transmission is the fecal–oral route, but its zoonotic potential is not completely understood. The objective of this study was to determine the presence and genetic diversity of *Blastocystis* on pig farms in Nakhon Pathom Province, Central Thailand. A total of 154 human and 90 pig stool samples were collected and analyzed. Nested PCR detected *Blastocystis* in 35.55% of the pig samples and 6.49% of the human samples. Subtyping based on regions of the small-subunit ribosomal RNA (SSU rRNA) gene identified three *Blastocystis* subtypes in pigs and humans: ST1, ST3, and ST5. *Blastocystis* ST5 was the predominant subtype, followed by ST1 and then ST3. All the sequences from the *Blastocystis*-positive samples from both pigs and humans were closely related. This study reveals a possibility of low host specificity of *Blastocystis* STs (ST1, ST3 and ST5) on pig farms in Thailand. We tentatively suggest that close contact with or exposure to pig stools may be a significant source of *Blastocystis* detected in pig handlers. Further studies are required to confirm the zoonotic transmission of this organism in Thailand, because pigs may play an important role in the transmission of *Blastocystis*.

1. Introduction

Blastocystis is a unicellular protist commonly found in humans and a variety of animals, including nonhuman primates, other mammals, and birds. It was first described more than 100 year ago, but its pathogenicity, mode of transmission, genetic diversity, host specificity, and treatment are still not well understood [1–5]. The clinical presentation of *Blastocystis* infection ranges from asymptomatic to symptomatic [6].

Blastocystis displays extensive genetic diversity, and 17 subtypes (STs) based on the small-subunit ribosomal RNA (SSU rRNA) gene have been observed in human and animal hosts [7]. These subtypes may be associated with distinct symptoms, risk factors, and zoonotic potentials [8]. ST1 to ST8 have been identified in humans and animals, whereas ST10–ST17 have only been observed in animal hosts [9]. Moreover, ST9 has only been identified from humans. Therefore, animals may act as the reservoir hosts of this organism, and zoonotic transmission may be one mode of *Blastocystis* infection in humans. Several risk factors are associated with *Blastocystis* infection, including poor personal hygiene, poor community sanitation, and close contacts with infected animals [10].

Several reports have demonstrated the prevalence and subtype distributions of *Blastocystis* (ST1, ST2, ST3, ST4, and ST5) in pigs [11–15]. Previous reports have demonstrated the prevalence and/or subtype distributions of *Blastocystis* in Thailand, in either humans or animals, but only a few studies have detected it in both humans and animals [11,16–19]. However, it has not been possible to identify the true sources of *Blastocystis* in the study areas. Consequently, it is difficult to eliminate or prevent *Blastocystis* infection in those areas. The objective of this study was to evaluate the presence and genetic diversity of *Blastocystis* in pigs in Thailand. Human and pig stool samples were collected from pig farms and communities near pig farms in Nakhon Pathom Province in central Thailand, which is one of the major pig-farming regions of the country. The prevalence and subtypes of *Blastocystis* were determined with molecular techniques. A phylogenetic

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analysis was performed to evaluate the subtype characteristics of this organism. The results of this study should support, improve, and promote better health and the quality of life in the study area, and the interactions between humans and animals.

2. Materials and methods

2.1. Study area and population

A cross-sectional study was conducted in Nakhon Pathom Province, Thailand, between December 2014 and March 2015. This Province is a region in the central part of Thailand, 56 km from Bangkok, and was selected because it is one of the major pig-farming Provinces in Thailand. We collected 90 pig stool samples from two pig farms (50 from pig farm no.1 and 40 from pig farm no.2) and 154 human stool samples, including samples from pig handlers (5 from pig farm no.1 and 4 from pig farm no.2) and individuals who lived near pig farms (radius about 50 to 300 m from the farm under study): 65 from pig farm no.1 and 80 from pig farm no.2. The specific instructions for collecting the stool samples and preventing sample contamination were clearly explained to all participants. The study protocol was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (MUTM 2014-051-01) and the Animal Care and Use Committee of the Faculty of Tropical Medicine, Mahidol University (FTM-ACUC 006/2014).

2.2. Molecular analysis

2.2.1. DNA extraction from stool samples

From each stool sample, 200 mg were used to DNA extraction using commercially available DNA extraction kit (PSP® Spin Stool Kit, Stratec Molecular, Berlin, Germany), according to the manufacturer's instructions.

2.2.2. Nested PCR analysis for Blastocystis

The small-subunit ribosomal RNA (SSU rRNA) gene was amplified from all the DNAs extracted from the stool samples, with nested PCR. Therefore, two sets of primers were used in this study. The first primer set for the first reaction was: forward primer: RD3 (5′-GGGATCCTGA TCCTTCCGCAGGTTCACCTAC-3′) and reverse primer: RD5 (5′-GGAAG CTTATCTGGTTGATCCTGCCAGTA-3′) [20]. The thermal cycling conditions were as follows: initial denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min, extension at 72 °C for 1 min, followed by final extension at 72 °C for 7 min. Each 25 μl reaction mixture contained 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM each primer, 2.5 U of *Taq* DNA polymerase (Fermentas, USA), 2 μl of genomic DNA extract, and 15.2 μl sterile distilled water. The second set of primers for the second reaction was: forward primer: forward B (5′-GGAGGTAGTGACAATAAATC-3′) and reverse primer: reverse B (5′-ACTAGGAATTCCTCGTTCATG-3′) [21]. The PCR cycling conditions were: initial denaturation at 95 °C for 4 min, 30 cycles of denaturation at 95 °C for 1 min, annealing at 54 °C for 1 min, extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. Each 25 μl reaction mixture contained 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM each primer, 2.5 U of *Taq* DNA polymerase (Fermentas, USA), 2 μl PCR products from the first reaction used as DNA templates, and 15.2 μl sterile distilled water. The PCR products (1100 bp) were separated on 1.5% agarose gel and visualized under UV transillumination.

2.3. Sequencing and phylogenetic analysis

All of positive PCR products from the second PCR reaction were purified before sequencing. All positive samples were sequenced with an ABI 3730xl sequencer, using fluorescent-dye terminator sequencing by Bio Basic Canada Inc. (Ontario, Canada). All the sequences of the *Blastocystis*-positive samples were determined for the highest similarity to the previously published sequences of ST1–ST17 in the GenBank database, using BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for subtype identification. Forty-two reference sequences of known *Blastocystis* subtypes ST1–ST17 (Table 1), the sequences of the *Blastocystis*-positive samples from this study, and two outgroup sequences (*Karotomorpha* sp. (DQ431242) and *Proteromonas lacertae* (U37108)) were aligned by MUSCLE and phylogenetic analysis was performed with MEGA version 6 [22]. The best model to account for the evolution of the DNA sequences was the Hasegawa–Kishino–Yano (HKY) + gamma distribution (G) + invariable (I) model. A phylogenetic tree was constructed using the maximum likelihood, neighbor-joining (NJ) and maximum parsimony (MP) methods with 1000 bootstrap replicates.

Table 1
GenBank references for the *Blastocystis* ST1–ST17 sequences used to construct a phylogenetic tree.

No.	Accession no.	Host	Subtype	Reference
1	U51151	Human	1	[30]
2	AB070989	Human	1	[31]
3	AB091241	Chicken	1	[31]
4	AF538349	Horse	1	[11]
5	AB107961	Pig	1	[32]
6	EU445492	Human	1	[13]
7	JQ665860	Human	1	[33]
8	JQ665863	Human	1	[33]
9	JQ665846	Human	1	[33]
10	JQ665867	Human	1	[33]
11	EU445487	Pig	2	[13]
12	AB107969	Pig-tailed macaque	2	[32]
13	EF200010	Human	2	[16]
14	AB070987	Human	2	[31]
15	AB070988	Human	3	[31]
16	AB107963	Pig	3	[32]
17	AB107965	Cattle	3	[32]
18	EU445494	Human	3	[13]
19	AB071000	Rat	4	[31]
20	AY244620	Human	4	[34]
21	U51152	Guinea pig	4	[30]
22	AB070998	Pig	5	[31]
23	AY266469	Toad	5	[34]
24	AB107964	Pig	5	[32]
25	AB107966	Cattle	5	[32]
26	EF468654	Human	5	Yan unpublished
27	EU445485	Chicken	6	[13]
28	JQ665850	Human	6	[33]
29	AB070991	Human	7	[31]
30	AY135412	Duck	7	[2]
31	AB107970	Lemur	8	[32]
32	AB107971	Pheasant	8	[32]
33	AF408425	Human	9	[34]
34	AF408426	Human	9	[34]
35	KC148207	Camel	10	[7]
36	GU256900	Elephant	11	[5]
37	GU256902	Giraffe	12	[5]
38	GU256935	Quokka	13	[5]
39	KC148205	Cattle	14	[7]
40	KC148210	Camel	15	[7]
41	EU427512	Red kangaroo	16	Yoshikawa unpublished
42	KC148208	Libya gundi	17	[7]

2.3.1. Nucleotide sequence accession numbers

The *Blastocystis* sequences identified in this study have been deposited in the GenBank database under accession numbers KT819588–KT819615, KT819620–KT819623, and MG333451–MG333456.

2.4. Statistical analysis

Descriptive analysis (percentages) was used to examine the positive stool samples and distributions of *Blastocystis* STs.