Am. J. Trop. Med. Hyg., 101(6), 2019, pp. 1392–1396
doi:10.4269/ajtmh.19-0569

Prevalence and Molecular Characterization of *Enterocytozoon bieneusi* among Pigs in Chonburi Province, Eastern Thailand

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Abstract. *Enterocytozoon bieneusi* is an organism that infects a wide variety of vertebrates, including humans. Pigs also harbor *E. bieneusi*, of which several genotypes have been recently detected in human feces. The aim of this study was to determine the prevalence of *E. bieneusi* infection among pigs raised in three smallholder farms and eight small large-scale farms in Chonburi Province, Eastern Thailand, using nested polymerase chain reaction of the internal transcribed spacer (ITS) of the small subunit of ribosomal RNA gene and to investigate genotypes of *E. bieneusi* isolates using nucleotide sequencing and phylogenetic tree analysis of the ITS region. Of 244 stool samples, *E. bieneusi* was detected in 14.8% (36/244). Two known zoonotic genotypes, that is, genotypes E (77.8%) and F (22.2%), were identified. Using phylogenetic tree analysis, these two genotypes were clustered in human pathogenic and zoonotic potential groups, designated as group 1. The high prevalence of zoonotic genotypes of *E. bieneusi* among pigs suggests that pig farming is one of the potential sources of human infection. This is the first report of *E. bieneusi* genotypes among pigs raised in pig farms in Eastern Thailand.

INTRODUCTION

Enterocytozoon bieneusi belongs to a group of spore-forming obligate intracellular parasites, Microsporidia, that infect vertebrates, including humans. This organism is known as an opportunistic intestinal parasite causing chronic diarrhea in immunocompromised hosts, especially among patients with HIV/AIDS.[1] The mode of transmission is mainly foodborne and waterborne.[2,3] Based on the polymorphism of the internal transcribed spacer (ITS) region of the small subunit of ribosomal RNA (SSU-rRNA) gene, more than 200 genotypes of *E. bieneusi* have been identified.[4] The zoonotic transmission between animals and humans has also been reported.[5] According to high prevalences of 10–94% of *E. bieneusi* infection reported in pigs, pigs have been considered one of the main reservoir hosts of *E. bieneusi*.[6–16] At present, approximately 40 genotypes of *E. bieneusi* have been reported in pigs, of which 13 genotypes are potentially zoonotic.[16] In Thailand, the information on the prevalence and genotypes of *E. bieneusi* among pigs remains limited.[17,18] Using the polymerase chain reaction (PCR) method, the studies of *E. bieneusi* infection among pigs in two provinces of Thailand showed prevalences of 13.7% in Nakorn Pathom and 41.7% in Kanchanaburi provinces. The identified genotypes were O, E, H, D, O, PigEbITS7, EbpA (F), and TMP1–TMP11.[17,18] In addition, the zoonotic genotypes identified among humans, genotypes E, D, O, and PigEbITS7, were also found among pigs.

A recent study showed that the pig industry in Thailand has been continuously growing over the last decades.[19] In terms of farm scales, pig farms in Thailand can be divided into smallholder farms (< 50 heads/farm) and large-scale farming systems (≥ 50 heads/farm) according to the number of pigs per farm. The large-scale farm systems were classified as small (50–500 heads/farm), medium (> 500–5,000 heads/farm), and large (> 5,000 heads/farm) farms. The majority of pig farms (95.02%) in Thailand were smallholders. However, large-scale farm systems contributed most of the pig stock (82%).[19] To promote healthy and hygienic pig-farming practices, the Agricultural Standard Committee, Ministry of Agriculture and Cooperatives, Thailand, has established the "Standard for Good Agricultural Practices for Pig Farms".[20] However, a recent report showed that a high percentage of small pig farms did not reach the criteria.[21]

To explore the transmission of *E. bieneusi* in pigs, a cross-sectional study was conducted in Chonburi Province, Eastern Thailand, where smallholder and small large-scale farms were located. The prevalence of *E. bieneusi* infection among pigs was investigated. In addition, genotypic characterization of *E. bieneusi* was performed using nested polymerase chain reaction (nPCR) of the SSU-rRNA gene, sequencing, and phylogenetic analysis of the ITS region.

MATERIALS AND METHODS

Specimen collection. In all, 244 fresh fecal specimens were collected from three smallholder and eight small large-scale pig farms in Chonburi Province, Eastern Thailand, from May 2015 to January 2016. All pig farms were managed under the open farm system with sanitary conditions. Strains of these pigs included three crossbreeds, that is, Large White, Landrace, and Duroc. To collect fecal samples in each pig farm, feces were randomly collected from up to 20% of the total number of pigs. Fecal samples were evacuated directly from the rectum using disposable gloves and placed in plastic containers. The age and sex of each pig were recorded during sampling. The specimens were classified in six age-groups (Table 1). All samples were kept in cool condition during transportation to the laboratory and then kept frozen at –20°C until DNA extraction.

Genomic DNA extraction, PCR amplification, and DNA sequencing of the SSU-rRNA gene. Genomic DNA was extracted from each stool sample using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and then kept at –20°C until further analysis. To identify *E. bieneusi*, the extracted DNA from

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TABLE 1

Characteristics of and prevalence of *Enterocytozoon bieneusi* infection among pigs raised in 11 farms

Characteristics		No. of samples	No. of positive samples (%)	*P*-value
Farm	1	21	1 (4.8)	0.402
	2	17	5 (29.4)	
	3	22	2 (9.1)	
	4	41	13 (31.7)	
	5	7	0	
	6	7	0	
	7	13	1 (7.7)	
	8	3	0	
	9	60	7 (11.7)	
	10	30	4 (13.3)	
	11	23	3 (13.0)	
Sex	Male	105	13 (12.4)	0.56
	Female	139	23 (16.5)	
Age (months)	≤ 2	24	2 (8.3)	0.152
	> 2–4	93	17 (18.3)	
	> 4–6	80	16 (20.0)	
	> 6–8	18	0	
	> 8–12	1	0	
	> 12	28	1 (3.6)	
Total		244	36 (14.8)	

Significant differences among groups were tested by chi-squared or Fisher's exact test.

each sample was subjected to amplify a fragment of the SSU-rRNA gene using nPCR. The nested primary amplification was conducted using specific primers and conditions described by Katzwinkel-Wladarsch et al.[22] The primary primers were MSP-1 (5′-TGA-ATG-KGT-CCC-TGT-3′) and MSP-2B (5′-GTT-CAT-TCG-CAC-TAC-T-3′), and the secondary primers were MSP-3 (5′-GGA-ATT–CAC-ACC-GCC-CGT-CRY-TAT-3′) and MSP-4B (5′-CA-AGC-TTA-TGC-TTA-AGT-CCA–GGG-AG-3′). All PCR reactions were conducted using 400 mM of each primer, 2 mM $MgCl_2$, and 200 mM of each deoxynucleoside triphosphate. The PCR cycling conditions consisted of an initial denaturation at 95°C for 10 minutes, followed by 35 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 10 minutes. The PCR products were gel electrophoresed using 1.5% agarose gel and visualized by gel documentation (Uvitech, Cambridge, United Kingdom).

The fragments of 530-bp PCR products of the SSU-rRNA gene were purified using the QAIquick Gel Purification Kit (Qiagen) according to the manufacturer's instructions. The purified PCR products were processed for bidirectional nucleotide sequencing using MSP-3 and MSP-4B primers by First BASE Laboratories, Malaysia.

Phylogenetic analysis of the ITS region. Chromatograms were manually checked and edited using BioEdit version 7.0.1 (Ibis Therapeutics, Carlsbad, CA).[23] The validated nucleotide sequences were compared with the relevant sequences of the *E. bieneusi* in GenBank using the BLAST. Nucleotide sequences in this study were assigned GenBank accession numbers as MN108201–MN108236. Subsequently, the ITS gene sequences (243 bp) were compared with other genotypes in the GenBank using BioEdit version 7.0.1. Phylogenetic analysis of about 243 bp of the ITS region was conducted using the neighbor-joining method in MEGA version 7.0.[24]

Statistical analysis. Data entry was conducted using Microsoft Excel (Microsoft, 2013). Data were analyzed using STATA/MP, version 12 (STATA Inc., College Station, TX). Descriptive analyses were carried out to determine characteristics of pigs and prevalence of *E. bieneusi* infection. The association between outcome and collected variables was analyzed by chi-squared or the Fisher's exact test. All statistical parameters were calculated with a *P*-value of 0.05.

RESULTS

In all, 244 pig fecal samples from 11 pig farms were collected in this study. Characteristics of pigs are shown in Table 1. Pig farm nos. 5, 6, and 8 were smallholder farms; the others were small large-scale farms. Thirty-six (14.8%) of 244 fecal specimens was positive for PCR product, revealing DNA band at about 530 bp. *Enterocytozoon bieneusi* was found in pigs collected from eight farms, with the exception of all smallholder farms (nos. 5, 6, and 8). The highest prevalence of infection was found in farm no. 4 (31.7%) followed by farm no. 2 (29.4%). Thirteen of 105 (12.4%) male and 23 of 139 (16.5%) female pigs were infected. Pigs aged between > 2 and 6 months showed a high prevalence of *E. bieneusi* infection. However, *E. bieneusi* was not detected in two age-groups, namely, > 6–8 and > 8–12 months. The prevalences of *E. bieneusi* infection in farms, sex, and age-groups did not significantly differ (Table 1).

Based on nucleotide sequence analysis of the ITS region, 28 of 36 (77.8%) and 8 of 36 (22.2%) samples were identified as genotypes E and F, respectively (Table 2). Genotype E was found in all positive farms (farm nos. 1, 2, 3, 4, 7, 9, 10, and 11), whereas genotype F was found in farm nos. 2, 4, 10, and 11. In addition, genotype E was found in male pigs at 10/13 (76.9%) consistency to female pigs at 18/23 (78.3%). *Enterocytozoon bieneusi* was commonly found in pigs in three age-groups, aged > 2–4 (17/17, 100%), > 4–6 (10/16, 62.5%), and > 12 (1/1, 100%) months. However, genotype F was commonly found among pigs aged ≤ 2 (2/2, 100%) and > 4–6 months (6/16, 37.5%).

In this study, phylogenetic analysis of the ITS sequences (Figure 1) showed that genotype E and F sequences were

TABLE 2

Genotypes of *Enterocytozoon bieneusi* isolated from pigs in 11 farms, Chonburi Province

Characteristics		No. of positive samples (%)	
		Genotype E	Genotype F
Farm	1	1/1 (100)	0
	2	3/5 (60)	2/5 (40)
	3	2/2 (100)	0
	4	9/13 (69.2)	4/13 (30.8)
	5	0	0
	6	0	0
	7	1/1 (100)	0
	8	0	0
	9	7/7 (100)	0
	10	3/4 (75)	1/4 (25)
	11	2/3 (66.7)	1/3 (33.3)
Sex	Male	10/13 (76.9)	3/13 (23.1)
	Female	18/23 (78.3)	5/23 (21.7)
Age (months)	≤ 2	0	2/2 (100)
	> 2–4	17/17 (100)	0
	> 4–6	10/16 (62.5)	6/16 (37.5)
	> 6–8	0	0
	> 8–12	0	0
	> 12	1/1 (100)	0
Total		28/36 (77.8)	8/36 (22.2)

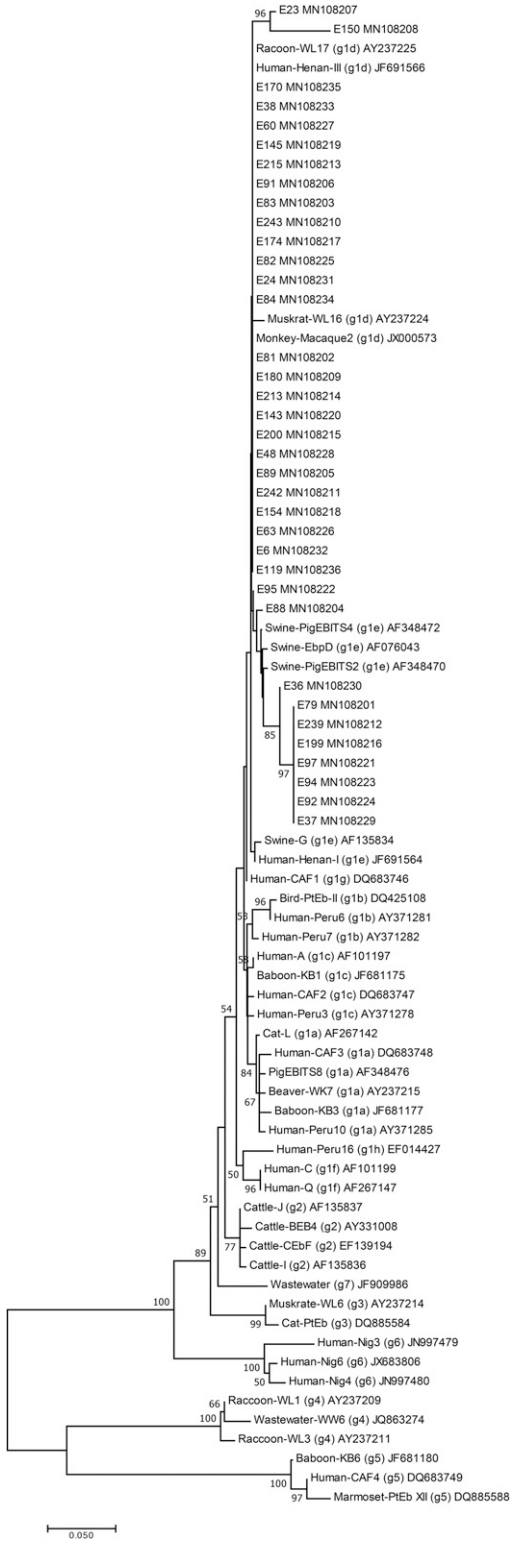

FIGURE 1. Phylogenetic relationships of the *Enterocytozoon bieneusi* genotypes identified in this study and other reported genotypes. The phylogeny was inferred with a neighbor-joining analysis of the internal transcribed spacer sequence based on distances calculated with the Kimura two-parameter model. The reliability of cluster formation was evaluated by the bootstraps method with 1,000 replicates.

clustered in group 1, which was previously described as human pathogenic and zoonotic potential groups.[25] All eight sequences of genotype F identified from pigs in the present study and the previous reported sequences, AF348472 (PigEBITS4), AF076023 (EbpD), and AF344470 (PigEBITS2), belonged to subgroup 1e. Moreover, all 29 sequences of genotype E from pigs in the present study and the previous reported sequences, AY237225 (Raccon-WL17), JF691566 (Human-Henan-III), AY237224 (Muskrat-WZ16), and JX000573 (Monkey-Macaque2), belonged to subgroup 1d.

DISCUSSION

In this study, the overall prevalence of *E. bieneusi* infection among pigs raised in 11 pig farms was 14.8%. A wide range of prevalences of *E. bieneusi* infection among pigs have been reported from many countries including Brazil, 59.3%[16]; the United States, 32%[9]; PR China, 16.4–45.1%[14,15]; the Czech Republic, 94%[11]; Switzerland, 34.9%[6]; Japan, 33.3%[13]; Germany, 10–66.7%[7,8,12]; and South Korea, 14%.[10] Compared with a related study in Thailand, a similar result was observed among pigs (15.7%) raised in pig farms in Nakorn Pathom Province, Central Thailand.[17] However, the prevalence was lower than that in Kanchanaburi Province, Western Thailand (42.6%).[18]

In this study, no significant differences were found in the prevalence of *E. bieneusi* infection among farms, age-groups, and sex of pigs ($P > 0.05$), and was similar to a related study conducted in the Czech Republic where no difference of *E. bieneusi* infection was found in each age-group of pigs.[11] However, a significantly higher prevalence of *E. bieneusi* infection was found in pigs aged 2–3.9 months than in those of other age-groups.[17] In PR China, the prevalence of *E. bieneusi* infection among pigs aged < 1 month (63.6%) was higher than that in pigs aged 1–2 months (41.0%) and > 2 months (26.3%).[15] The difference of prevalence rates in age-groups of pigs in each study area may be due to the overall environmental hygiene of the farm, farm management systems, quality of drinking water, and intensity of pigs in the farms. In this study, the prevalence of *E. bieneusi* infection among 11 pig farms did not significantly differ. However, *E. bieneusi* infection was detected in eight small large-scale farms, but not in three smallholder farms. All pigs aged between 6 and 12 months that had no infections were from these three smallholder farms. The number of pigs that might represent the density in each farm could explain this finding.

Several zoonotic genotypes have been reported from pigs worldwide, namely, BEB4, CAF1, D, I, O, PigEbITS7, EbpD, EbpA (F), EbpC (E), PigEbITS5, LW1, Henan-III, and Henan-IV.[16] Among these, genotypes E, H, O, PigEbITS7, D, and F were identified in pigs in Thailand.[17,18] In this study, genotype E was the most numerous (77.8%) found in pigs, as well as 57% reported in a related study.[17] In addition, 15% of genotype E was also detected among humans in Bangkok.[26] Moreover, using phylogenetic analysis, genotypes E and F are clustered in group 1, suggesting the possibility of zoonotic transmission and public health significance. However, other groups (groups 2–8) are found in specific hosts and wastewater collected in the environment.[25,27] In this study, these smallholder pig farms were located close to human residences, which could be the source of transmission of zoonotic genotypes of *E. bieneusi* to people at risk, especially those who are HIV positive or who are otherwise immunodeficient.

Genotypes E and F of *E. bieneusi* among pigs are distributed worldwide, which have been reported in Japan,[13] the Czech Republic,[11] Germany,[7,8,12] Switzerland,[6,28] the United States,[9] Brazil,[16] and PR China.[15] Indeed, these two genotypes have been reported in a wide range of animals such as dogs,[27] goats,[29] cattle,[7] and beavers and muskrats.[6] Therefore, genotypes E and F have broad host specificity, and can be transmitted from animals to humans. In our study, a majority of the animals shedding genotypes E and F were weaning pigs (aged > 2–4 months) and adult pigs (aged > 4–6 months), respectively. It appears that these age-groups of pigs are an important reservoir harboring zoonotic genotypes of *E. bieneusi*.

Pigs infected with *E. bieneusi* at an early age can excrete infected spores lifelong.[11] In general, most infected pigs are asymptomatic. In Thailand, a few pig farms were integrated with other livestock, crops, vegetables, and fruit production. Pig waste can be used to produce organic fertilizer for plants. This could cause animal to animal and animal to human transmission. A large number of spores among animals can also enter the environment and cause wider geographical spread of *E. bieneusi* spores. Understanding the molecular epidemiology of *E. bieneusi* in different hosts is an important step in adequately controlling *E. bieneusi* infection among humans because of the lack of an effective vaccine and available drugs.

In conclusion, the prevalence of *E. bieneusi* was 14.8% among pigs raised in smallholder pig farms in Eastern Thailand. Two genotypes, E and F, were detected and were clustered in group 1, suggesting pigs could be a potential source of *E. bieneusi* infection to humans. The "Standard for Good Agricultural Practices for Pig Farms" established by the Agricultural Standard Committee, Ministry of Agriculture and Cooperatives, Thailand, should be promoted to limit their potential health impact.

Received July 30, 2019. Accepted for publication August 22, 2019.

Published online September 23, 2019.

Acknowledgments: This work was financially supported by a Research Grant from Burapha University through the National Research Council of Thailand (grant nos. 100/2559 and 121/2560).

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