Molecular detection and genotyping of *Blastocystis* and *Enterocytozoon bieneusi* in humans and pigs in Nakhon Pathom Province, Thailand

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Abstract

Blastocystis, *Cryptosporidium* spp. and *Enterocytozoon bieneusi* are enteric protozoa in a wide range of vertebrate hosts, causing diarrheal diseases. There is scanty molecular data on their frequency among humans and animals in Thailand. In this study, a total of 142 human and 102 pig stool samples were collected from pig farms in Nakhon Pathom Province and evaluated for the presence of the protozoa by nested PCR. In human, 2.8% of *Blastocystis* and 9.2% of *E. bieneusi* were found while 20.6% of *Blastocystis* and 15.7% of *E. bieneusi* were found in pigs. No *Cryptosporidium* spp. (0%) was detected in both pig and human samples. Four subtypes of *Blastocystis* were identified and the zoonotic potential in this was possibly due to direct or indirect contact of humans to pig feces. Two previously reported genotypes of *E. bieneusi* and 6 new genotypes were identified in pig. In humans, 3 previously reported and 7 new *E. bieneusi* genotypes were identified. The significance of the present results highlights that it is crucial to strength surveillance on intestinal pathogens as close contact between humans and pigs may increase the risks of zoonotic transmission, especially from *Blastocystis* and *Enterocytozoon*.

Key words: *Blastocystis / Enterocytozoon bieneusi / Cryptosporidium*

Introduction

Blastocystis, Enterocytozoon and *Cryptosporidium* are intestinal protozoa found in humans and animals. These organisms can cause intestinal symptoms such as diarrhea in both immunocompromised and immunocompetent patients (Santin and Fayer, 2011; Tan, 2008; Xiao, 2010). The transmission routes are fecal-oral, water-borne, and food-borne. Moreover, zoonotic transmission between humans and a variety of animals is common. In farm areas, zoonotic transmission is important due to the high opportunity with the animal contact such as domestic animals (Daniels et al., 2015; Schar et al., 2014). Diagnosis of intestinal protozoa are usually based on direct fecal examination by microscopy. However, the sensitivity of this method is low and requires an expertized microscopist. Culture methods are more sensitive than direct examination but time-consuming and not routinely performed in many laboratories. Molecular technique such as PCR is more sensitive, rapid and also useful for genotyping and subtyping these organisms. It is widely used in epidemiological studies in clinical samples recently (Abu Samra et al., 2012; Popruk et al., 2015; Zebardast et al., 2016).

Pigs are one of the most important domestic animals for consumption. In Thailand, the pork consumption rate was 13.7 kg per capita and this trend has been increasing since 1999 (Sutthikornchai et al., 2013). The risk of zoonotic transmission in animal handlers have a significantly higher than individuals who do not work with animals and it had been suspected that some protozoan isolates from animals have zoonotic potential (Abe et al., 2003). The objective of this study was to determine the prevalence, and genosubtypes of the intestinal protozoa (*Blastocystis, Enterocytozoon* and *Cryptosporidium*) among pigs and human in Thailand. Nakhon Pathom Province was selected because many of pig farms are located near the villages. The investigation data obtained from this study may pinpoint and resolve issues related to enteric/food and waterborne protozoa, leading to effective interventions to improve morbidity and mortality due to enteric/food and waterborne diseases. Moreover, such a study may be a good model for other Provinces where pigs and humans live close to each other.

Materials and methods

A total of 102 pig stool samples collected from 2 pig farms in Phai Hu Chang Sub district, Bang Len District, Nakhon Pathom Province and 142 human stool samples collected from villagers (male:female ratio, 55:87; median age, 41 years; age range, 18 years to 67 years) who live near the farms were included in this study. Approximately 200 mg of each fecal sample was extracted DNA with the PSP Spin Stool DNA Kit (Stratec Inc., Germany) according to the manufacturer's instructions. *Blastocystis* and *Cryptosporidium* were detected with the PCR amplification of the small subunit (SSU) rRNA. *E. bieneusi* was screened with the PCR amplification of the SSU rRNA ITS gene. The primer sets and conditions were used according to previously published articles (Ryan et al., 2003; Sulaiman et al., 2003; Wong et al., 2008). PCR products were detected and visualized by electrophoresis. All amplified products were sequenced in both directions using the secondary PCR primers on an ABI 3730xl DNA analyser (Applied Biosystems, Foster City, CA, USA). The genotypes/subtypes from each specimen were confirmed by homology of sequenced PCR products to the sequence published in GenBank. Nucleotide sequences of novel *E. bieneusi* isolates in the present study will be deposited in GenBank. The study protocol was approved by the Ethics Committee at the Faculty of Tropical Medicine, Midol University (MUTM 2014-051-01), as well as the Faculty of Tropical Medicine-Animal Care and Use Committee, Mahidol University (FTM-ACUC 006/2014).

Results

Analysis of the 244 collected fecal samples showed that 4 humans (2.8%) and 21 pigs (20.6%) were infected with *Blastocystis*. *Cryptosporidium* spp. was not detected in both pig and human. *E. bieneusi* were detected in 13 (9.2%) of human samples and 16 (15.7%) of pig samples (Table 1). In pigs, *Blastocystis* subtype (ST) 5, and *E. bieneusi* genotype H and E were the most prevalent while in humans, *Blastocystis* ST1, ST3 were found, and *E. bieneusi* genotype TMH2 were the most prevalent. Phylogenetic analysis of *Blastocystis* isolates from pig and humans in Nakhon Pathom Province is shown in Figure 1.

Table 1: The number of positive samples, subtypes of *Blastocystis* and genotypes of *Enterocytozoon bieneusi* in this study.

Host (No. of sample)	Subtype or genotype (positive samples) (zoonotic transmission)	
	Blastocystis	*E. bieneusi*
Humans (142)	ST1 (1); ST3 (3) (Zoonotic)	D (1); H (2); EbpD (1) (Zoonotic potential)
		TMH2 (3); TMH3 (1); TMH4 (1); TMH5 (1); TMH6 (1); TMH7 (1); TMH8 (1) (Unclear zoonotic transmission)
Pigs (102)	ST1 (4); ST3 (1); ST5 (16) (Zoonotic)	H (4); E (4) (Zoonotic potential)
		TMP6 (2); TMP7 (1); TMP8 (1); TMP9 (1); TMP10 (2); TMP11 (1) (Unclear zoonotic transmission)
Total (244)	25 (10.2%)	29 (11.9%)

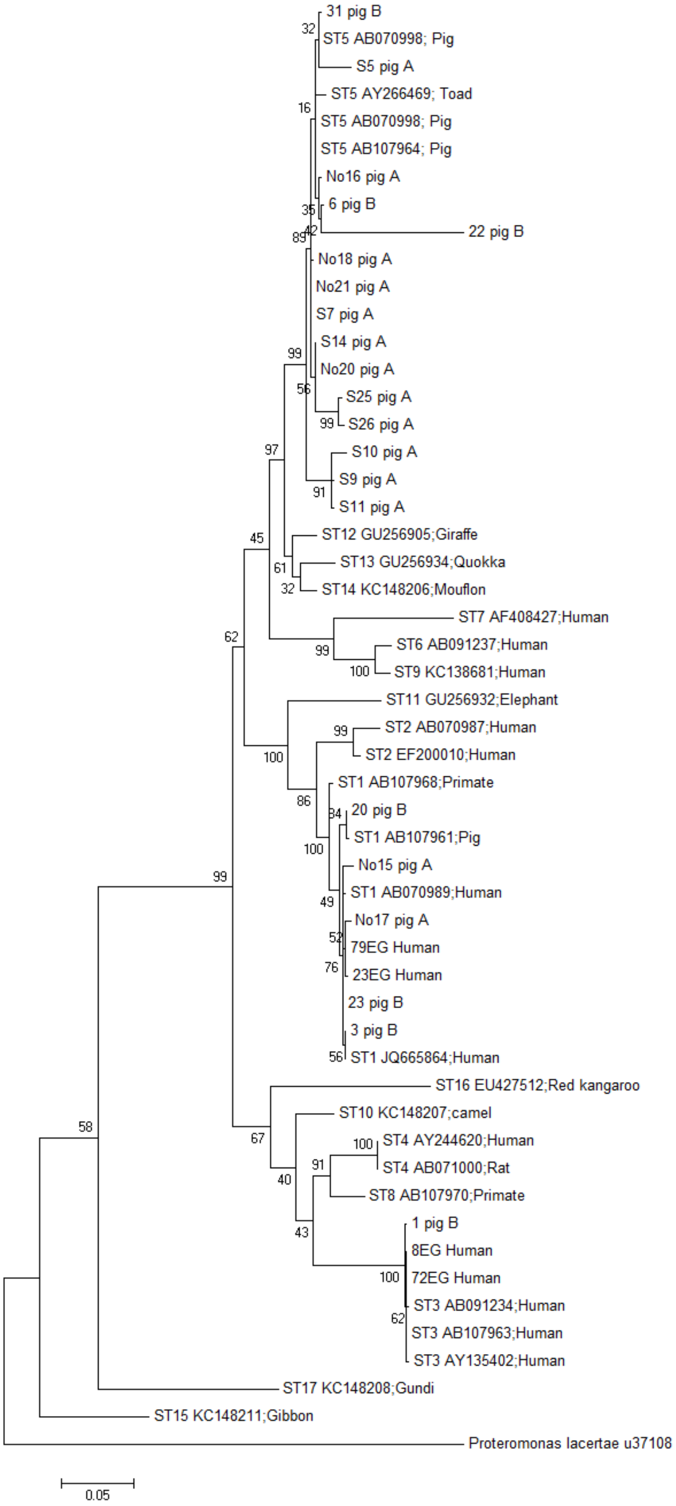

Figure 1 Phylogenetic analysis of *Blastocystis* isolates from pig and humans in Nakhon Pathom Province. The Maximum likelihood phylogenetic tree of *Blastocystis* isolates from pig and humans in Nakhon Pathom Province inferred from SSU rRNA gene sequences is shown.

Discussion

To date, at least 17 distinct STs of *Blastocystis* have been identified in humans, non-human primates, mammals, and avian hosts, based on the analysis of the SSU rRNA genes of which ST 1-9 have been reported in humans with ST1-ST4 are most common while ST 10-17 have been reported from animals only (Popruk et al., 2015). The prevalence of 2.8% *Blastocystis* in humans in this study was similar to a previous study (5.9%) in communities along the Chao Phraya River (Palasuwan et al., 2016) but lower the prevalence in Kanchanaburi Province (12.82%) (Udonsom et al., 2018). The low prevalence in this study might indicate a good parasite prevention or a good waste management in farms. On the other hand, a relatively high prevalence of *Blastocystis* infection (37.2%) was found in Thai-Myanmar Border communities where personal hygiene and waste management were very poor (Popruk et al., 2015). Overall, the prevalence in our study was much lower than children (40.7%), workers (28.6%) and household members (50%) in Brazil that investigated *Blastocystis* in human populations living in poor and deprived areas (Oliveira-Arbex et al., 2018).

The present study showed similar low prevalence of *Cryptosporidium* in pigs compared to previous surveys conducted in China (Lin et al., 2015). This coincides with the findings of Wieler and colleagues who reported 1.4% of infection (Wieler et al., 2001). Many factors have been considered to influence the prevalence of pig cryptosporidiosis among different areas and countries, including pig health status, age categories, breeding mode, and management style. However, it is unclear why both pigs and humans are not infected with *Cryptosporidium* in this study.

The prevalence of *E. bieneusi* in humans (9.2%) was higher than children in rural communities (3.8%) (Mori et al., 2013) and higher than humans in pig farm in central areas (1.4%) (Leelayoova et al., 2009). From previous studies in Thailand, the prevalence of *E. bieneusi* was highest in HIV patients (33.3%) (Wanachichwanawin et al., 1998). In pigs, the prevalence (15.7%) was similar to pig farms in central area (Leelayoova et al., 2009). *E. bieneusi* was found in cat (31.3%) in a temple in central Thailand. When compare to other countries, the prevalence in China was higher with 83.2% (Zhao et al., 2014), 45.1% in northeast China (Li et al., 2014) and 59.3% in Brazil (Fiuza et al., 2015). *E. bieneusi* zoonotic genotypes have been identified from all of the settings. The predominant genotypes found in human was H followed by D (equal to EbpD), and novel genotype TMH2-TMH8 were identified.

In summary the zoonotic risk of *Blastocystis* in Nakhon Prathom was not high. Although the prevalence of *Blastocystis* in pigs was 20.6%, the prevalence of *Blastocystis* in human was low (2.8%) indicating that both pig farms had satisfactory waste management and good personal hygiene to prevent transmission of *Blastocystis*. However, pigs may play a major role in the transmission of *Blastocystis* and *E. bieneusi* to humans as zoonotic subtypes and genotypes were found. Further studies of *Blastocystis* and *E. bieneusi* in pigs are necessary to evaluate the genotypic characteristics, geographical distribution, pigs are possible role as reservoir hosts, and potential risks for humans.

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