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Molecular epidemiology of *Blastocystis* in pigs and their in-contact humans in Southeast Queensland, Australia, and Cambodia

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ABSTRACT

Blastocystis, an intestinal protist commonly found in humans and animals worldwide, has been implicated by some as a causative agent in irritable bowel syndrome in humans. In pigs, infection with *Blastocystis* is commonly reported, with most pigs shown to harbour subtypes (ST) 1 or 5, suggesting that these animals are potentially natural hosts for Blastocystis. Although ST5 is considered rare in humans, it has been reported to be a potential zoonosis from pigs in rural China. To test these hypotheses, we conducted molecular analysis of faecal samples from pigs and in-contact humans from commercial intensive piggeries in Southeast Queensland (SEQ), Australia, and a village in rural Cambodia. The prevalence of *Blastocystis* in SEQ and Cambodian pigs was 76.7% and 45.2%, respectively, with all positive pigs harbouring ST5. It appears likely that pigs are natural hosts of *Blastocystis* with a high prevalence of ST5 that is presumably the pig-adapted ST in these regions. Amongst the SEQ piggery staff, 83.3% were *Blastocystis* carriers in contrast to only 55.2% of Cambodian villagers. The predominant STs found in humans were STs 1, 2 (Cambodia only) and 3. Interestingly, ST5 which is usually rare in humans was present in the SEQ piggery staff but not in the Cambodian villagers. We conclude that in intensive piggeries, close contact between pigs and their handlers may increase the risks of zoonotic transmission of *Blastocystis*.

1. Introduction

Blastocystis is an intestinal protist that is commonly found worldwide in a diverse range of species including humans, non-human primates (NHPs), other mammals and birds (Alfellani et al., 2013c; Stensvold et al., 2009). *Blastocystis* is genetically polymorphic and molecular analysis of the small subunit ribosomal RNA (SSU rRNA) gene has enabled division of this organism into 17 distinct subtypes (STs) found in a wide range of species (Alfellani et al., 2013c; Fayer et al., 2012; Parkar et al., 2010; Stensvold et al., 2009). Some STs show significant intra-ST genetic variability (Scicluna et al., 2006; Stensvold et al., 2012; Yoshikawa et al., 2009). Humans have been shown to carry STs 1–9 with ST3 the most frequently reported. Significant geographical variation in the prevalence and relative proportion of STs is known to exist, with other common STs including 1, 2 and 4 (Alfellani et al., 2013b). Faecal

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oral transmission either by direct contact or water-borne transmission is currently the most accepted mode of transmission (Lee et al., 2012b; Leelayoova et al., 2004, 2008). Previous studies have isolated identical STs of *Blastocystis* from humans and their in-contact animals including pigs (Lee et al., 2012a,b; Nagel et al., 2012; Parkar et al., 2007, 2010; Stensvold et al., 2009; Yan et al., 2007), suggesting potential zoonotic transmission of *Blastocystis*.

Blastocystis has been reported in pigs in some countries, with most pigs harbouring ST5 or ST1 and occasionally ST2 or ST3 (Alfellani et al., 2013c; Navarro et al., 2008; Thathaisong et al., 2003; Yan et al., 2007). In Valencia, Spain, Navarro et al. (2008) found ST1 to be the most prevalent among intensively reared pigs and, to a lesser extent, ST2. ST5 has also been identified in cattle, other livestock and captive apes (Alfellani et al., 2013b; Stensvold et al., 2009), but rarely in humans. The potential of pigs to act as zoonotic reservoirs of *Blastocystis* was demonstrated by Yan et al. (2007) in which two human ST5 isolates had restriction fragment length polymorphism (RFLP) patterns of the SSU rDNA were either identical or similar to those of 16 pigs living in the same rural area in China.

The objective of this study was to ascertain if pigs are suitable animal models for studying *Blastocystis* infection by investigating if they are indeed natural hosts of *Blastocystis* as suggested by previous reports. Our selection criteria for a 'natural host' was based on (i) a host which commonly harbour *Blastocystis* (i.e. at a high prevalence) and (ii) of a predominant ST or STs. The second objective was to investigate the zoonotic potential of porcine *Blastocystis* by testing and genetically comparing *Blastocystis* recovered from pigs and their in-contact humans (i.e. piggery staff and villagers).

Preceding studies investigating *Blastocystis* infection in pigs have been limited by their relatively small sample size and lack of molecular identification of *Blastocystis* ST (Abe et al., 2003; Navarro et al., 2008; Yan et al., 2007). In this study we genetically characterise the STs of *Blastocystis* in pigs and their in-contact humans in two different settings (intensive versus small holder piggeries) in Southeast Queensland (SEQ), Australia, and a rural Cambodian village.

2. Materials and methods

2.1. Sampling

2.1.1. Southeast Queensland (SEQ)

Single faecal samples were collected from 240 pigs and 36 piggery staff from 18 commercial piggeries in SEQ between February 2012 and February 2013 and stored in 2.5% potassium dichromate prior to DNA extraction. The faecal samples were collected from approximately 10–25 pigs at various stages of production (piglets, weaners, growers and sows) per piggery.

2.1.2. Cambodia

Single faecal samples were collected in May 2012 from 73 pigs and 210 villagers from 41 households in Dong village, Preah Vihear province, Cambodia and stored in 2.5% potassium dichromate prior to DNA extraction. The majority of the households sampled (29/41) owned pigs and up to 0–4 pig and 1–9 human samples were collected per household.

2.1.3. Ethical approval

The SEQ segment of this project was approved by the University of Queensland Animal Ethics Committee and Medical Research Ethics Committee with approval no. ANRFA/472/11 and 2012000069, respectively. Sample collection from the Cambodian village was made possible by collaboration between the University of Copenhagen, Denmark, Department of Fisheries Post-Harvest Technologies and Quality Control, Cambodia, and the National Center for Parasitology, Entomology and Malaria control, Cambodia. The study protocol was approved by the National Ethics Committee Health Research, Ministry of Health in Cambodia. All persons were informed on the purpose of the study. Written informed consent was obtained from all individuals prior to enrolment.

2.2. Molecular analysis

2.2.1. DNA extraction

Faeces stored in 2.5% potassium dichromate was washed twice in 1× phosphate-buffered saline and centrifuged before the sediment was used for DNA extraction. DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Germany) with minor alterations as per Wang et al. (2013).

2.2.2. PCR amplification with nested PCR

Samples were first tested with a previously published nested PCR primer set and conditions that amplified an 1100 bp region of the *Blastocystis* SSU rRNA gene (Clark, 1997; Wong et al., 2008) (Table 1). This method was chosen since it provides a large fragment for phylogenetic analysis, and as it is a pan-*Blastocystis* technique, it allows for identification of known and unknown STs of *Blastocystis*. Each 25 μl PCR reaction was run using approximately 50 ng of DNA and the conditions were as per Clark (1997) and Wong et al. (2008).

2.2.3. Phylogenetic analysis

All positive PCR products were purified using the PureLink Genomic DNA Mini Kit (Life Technologies Corporation, New York, USA) according to the manufacturer's protocol. Unidirectional DNA sequencing was performed with the reverse primer of the secondary PCR with an Applied Biosystems 3130/3130*xl* Genetic Analyzer. The DNA sequences were first analysed with Finch TV v 1.4.0 (Geospiza Inc., Seattle, WA, USA) and subsequently compared with published sequences from GenBank (National Center for Biotechnology Information) using BLAST 2.2.9 (http://blast.ncbi.nlm.nih.gov/Blast.cgi). These sequences were then aligned with previously published sequences of the SSU rRNA gene of the various *Blastocystis* STs, which were sourced from GenBank using BioEdit v 7.1.3.0 software (Ibis Biosciences, Carlsbad, CA, USA). The sequences were subsequently analysed using Mega 4.1 software (The Biodesign Institute, Tempe, AZ, USA) to construct a neighbour joining tree for subtyping of the *Blastocystis* isolates. *Proteromonas lacertae* (U37108) was used as an out-group.