PREVALENCE, ULTRASTRUCTURE AND SUBTYPES OF *BLASTOCYSTIS* IN CHICKENS (*GALLUS GALLUS*) FROM PENINSULAR MALAYSIA

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Abstract. The frequent consumption of chicken products in Malaysia raises the concern about the risk for contracting *Blastocystis* infection from this consumption. We aimed to investigate the current prevalence, ultrastructure and subtypes of *Blastocystis* sp isolated from feces of free-range and barn-reared chickens in Peninsular Malaysia. Fresh fecal samples were collected and examined microscopically and cultured in Jones' medium supplemented with 10% horse serum. The infection rate was 26.2% (47 chickens were positive for *Blastocystis* out of 179 chickens) with significantly higher infection rate observed in the free-range chickens (34.3%; 36/105) compared to the barn-reared population (14.9%; 11/74) (p=0.004). The most common form of the organism seen on culture was vacuolar form. The sizes seen on culture ranged from 10 μm to 100 μm. The cell bodies stained with Sudan Black B were positive for lipids. On examination with electron microscopy, smooth surface and electron-dense central vacuoles were seen in the cell bodies of those isolated from the free-range chickens but not the barn-reared chickens which consists of coarse surface and electron-opaque central vacuole. *Blastocystis* isolates were subsequently subjected for DNA barcoding method and four subtypes were identified. This is the first published study to evaluate the prevalence, surface structure, ultrastructure and subtypes of *Blastocystis* sp isolated from free-range and barn-reared chicken in Malaysia.

Keywords: *Blastocystis*, DNA barcoding, poultry, subtypes, zoonotic

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INTRODUCTION

Blastocystis, a stramenopile (Silberman *et al*, 1996), can infest the gastrointestinal tract of humans and many animals (Abe *et al*, 2003). Fecal-oral transmission is the most common transmission pathway

with the cystic form being the transmissible life cycle stage (Tan, 2004). *Blastocystis* infections have been reported to occur in turkeys (Lee, 1970), ostriches (*Struthio camelus*) (Yamada *et al*, 1987; Ponce Gordo, 2002; Hemalatha *et al*, 2014; Chandrasekaran *et al*, 2014), domestic chickens (*Gallus gallus*) (Yamada *et al*, 1987; Belova, 1990; Bergamo do Bomfim *et al*, 2013) and domestic ducks (*Anas platyrhynchos f. domestica*) (Pakandl and Pecka, 1992). To date, three *Blastocystis* subtypes have been identified in chickens: ST1 (Yoshikawa *et al*, 1996; Yoshikawa *et al*, 2003; Abe, 2004), ST6 (Yoshikawa *et al*, 2003; Arisue *et al*, 2003) and ST7 (Noël *et al*, 2003; Abe, 2004; Alfellani *et al*, 2013) with vacuolar forms being the forms most commonly observed (Yamada *et al*, 1987; Bergamo do Bomfim *et al*, 2013).

Chicken meat is the most popular and cheapest meat protein among Malaysians (Ministry of Agriculture and Agro-based Industry, 2011) because there are no dietary prohibitions or religious restrictions against chicken consumption. This led us to investigate the prevalence of *Blastocystis* in the Malaysia chicken population between different methods of raising the chicken: free-range or barn-reared chickens, to describe the ultrastructure of the isolates and identify their subtypes. We also compared infections between different farming practices - free-range and barn-reared chickens - as there have been no attempts to date to substantiate claims that indoor reared animals in regulated temperatures with proper ventilation and subjected to antibiotic treatments are free from *Blastocystis* infections.

MATERIALS AND METHODS

Study animals

We screened free-range and commercial, barn-reared chicken for *Blastocystis* infection. All the animals used in this study were handled following the guidelines of the Institutional Animal Care and Use Committee (IACUC), University Malaya (No.: ISB/31/01/2013/SNMZ (R)). Permission to conduct the study was obtained from the Ipoh City Council and the Kuala Lumpur City Hall. Ethical approval for the study was obtained from the Ethics Committee from the Faculty of Science, University of Malaya.

Free-range chickens

The free-range chickens are released daily, allowed to scavenge for food freely, and return periodically to the homestead for water and to feed on kitchen refuse. Nowadays, they become popular in rural households where there is an emerging trend of consumer awareness towards organically grown chickens, with customers increasingly willing to pay high prices for good quality meat.

A total of 105 fecal samples of free-range chicken were collected from two locations: Kinta, Perak and Kuala Selangor, Selangor, Malaysia with additional free roaming chickens from a mini zoological park located in northern Perak. Fecal sampling off the ground was conducted individually and the samples were cultured as soon after collection.

Barn-reared chickens

The barn-reared chickens consisted of commercial broiler chickens reared specifically for meat. The birds were kept indoors, with regulated temperature and ventilation either on deep litter (*eg*, wood shavings) or floors. The water provided to the chickens was sourced from either the municipality or pumped from nearby ponds.

Screening for *Blastocystis*

Fecal samples were collected from the rectum or base of each chicken and stored

in stool collection containers. The samples were then cultured using Jones' medium supplemented with 10% horse serum following the method of Kumarasamy *et al* (2014). After 24 hours, bottom of the culture medium was examined under a microscope at 400x and 1,000x magnification.

The isolates were then stained using Sudan Black B Staining System (Sigma Aldrich, Taufkirchen, Germany) according to the recommendations of the manufacturer (Chandrasekaran *et al*, 2014). The presence of black droplets in the central vacuole indicates positive reactions.

Ultrastructure screening

Selected isolates were examined for ultrastructure and surface structure using scanning and transmission electron microscopy following the method of Tan and Suresh (2006).

DNA examination

Genomic DNA was extracted from all *Blastocystis* isolates using QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) following to the manufacturer's instructions. A *Blastocystis*-specific primer, BhRDr (GAGCTTTTTAACTGCAACAACG; Scicluna *et al*, 2006) was paired with eukaryote-specific primer, RD5 (ATCTGGTTGATCCTGCCAGT; Clark, 1997) and used, in a single step PCR reaction, to amplify a 600 bp region of 18S rRNA. The PCR was performed in 25 μl of 1.0 mM dNTPs, 0.5 mM each primer, 1 x PCR buffer, 2.5 mM $MgCl_2$,1 U Taq DNA Polymerase (recombinant) (FERMENTAS, Waltham, MA) and 5 μl genomic DNA. The PCR was conducted as follows: denaturation at step of 94°C for 1 minute; followed by 30 cycles at 94°C for 1 minute each, 59°C for 1 minute and 72°C for 1 minute; followed by a final elongation step of 72°C for 2 minutes (Thermo Cycler Bio-Rad, Hercules, CA). PCR products were visualized on 1.5% agarose gel prior to purification; sequencing was performed at 1st Base, Selangor, Malaysia. Sequencing was checked using Seq Scanner 2 software (Applied Biosystems, Foster City, CA) for quality and subsequently were edited to remove low quality bases and primer sequences using BioEdit software (Carlsbad, CA). The edited sequences were then compared to a *Blastocystis* 18S rRNA database (http://www.pubmlst.org/*Blastocystis*) (Roberts *et al*, 2013; Farah Haziqah *et al*, 2018).

Statistical analysis

Chi-square analysis was carried out to determine any association between *Blastocystis* prevalence and farming practices, using Statistical Package for the Social Science (SPSS) version 21.0 (IBM, Armonk, NY). A p-value <0.05 was considered statistically significant.

RESULTS

Prevalence of *Blastocystis*

Twenty-six point two percent (47/179) of chicken fecal samples were positive for *Blastocystis*. The prevalence of *Blastocystis* infection was significantly (p=0.004) higher among free-range chickens (34.3%; 36/105) than barn-reared chickens (14.9%; 11/74). None of the *Blastocystis* infected chickens were symptomatic.

Morphological characteristics

Blastocystis isolated from free-ranged and barn-reared chickens were morphologically similar to *B. hominis*. Four forms of *Blastocystis* were observed: vacuolar and granular form. The vacuolar forms were the most commonly seen measuring approximately 10 μm to 100 μm in diameter (Fig 1a) whereas the granular forms size ranges from 10 to 90 μm in diameter (Fig 1b). Positive reactions

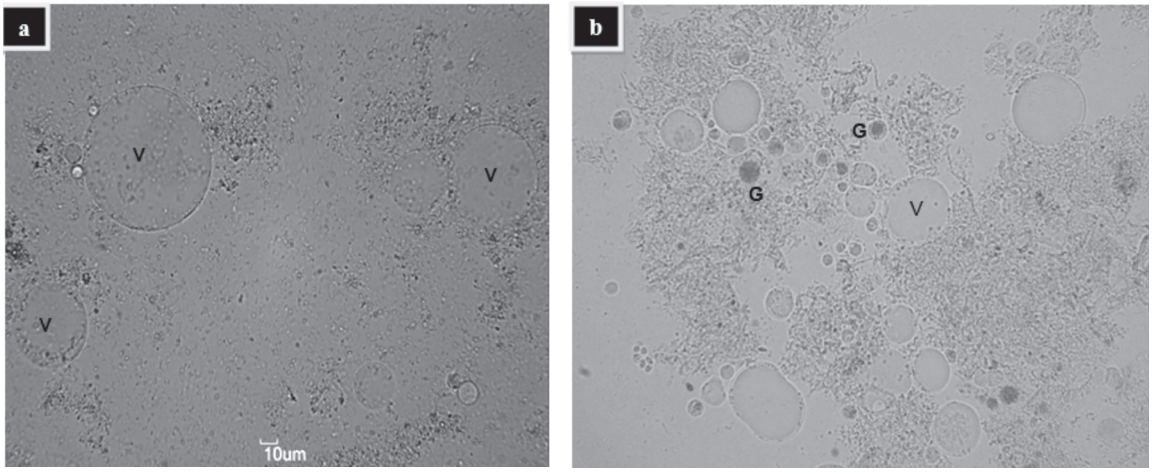

Fig 1 - Light micrographs showing *Blastocystis* isolated from (a) free-range chicken and (b) barn-reared chicken. V, vacuolar form; G, granular.

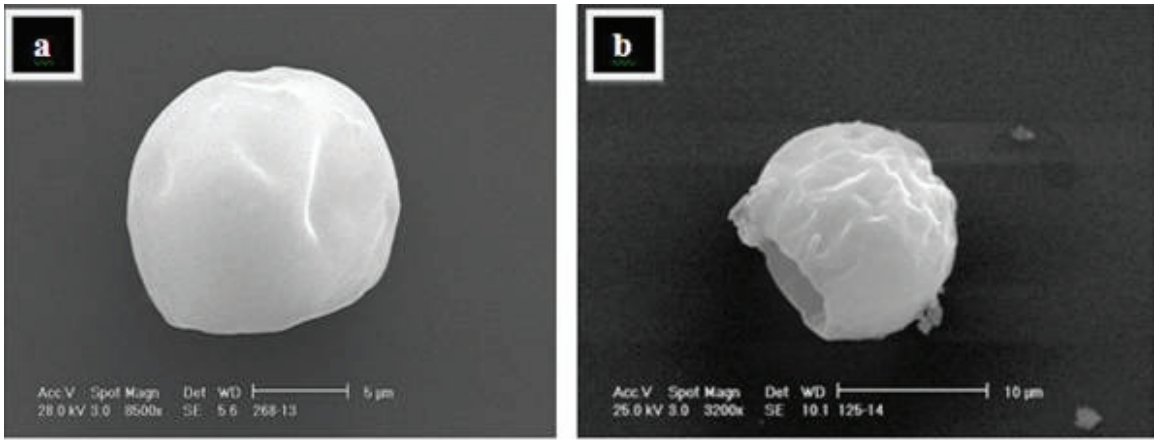

Fig 2 - Scanning electron micrographs showing *Blastocystis* isolated from (a) free-range chicken and (b) barn-reared chicken.

were observed in the central vacuole of *Blastocystis* sp isolated from barn-reared chickens stained with Sudan Black B.

Surface structure and ultrastructure

The isolates from free-range chickens possessed a smooth surface structure (Fig 2a) and a large central, completely electron-lucent central vacuole (Fig 3a). Meanwhile, the isolates from barn-reared chickens showed coarse with folds surface structure (Fig 2b) with electron-opaque, and fully distended central vacuole (Fig 3b).

Subtype identification

Of the 47 *Blastocystis* isolates found in our study, we were able to identify the subtype in ten isolates. The others could not be identified due to low number of

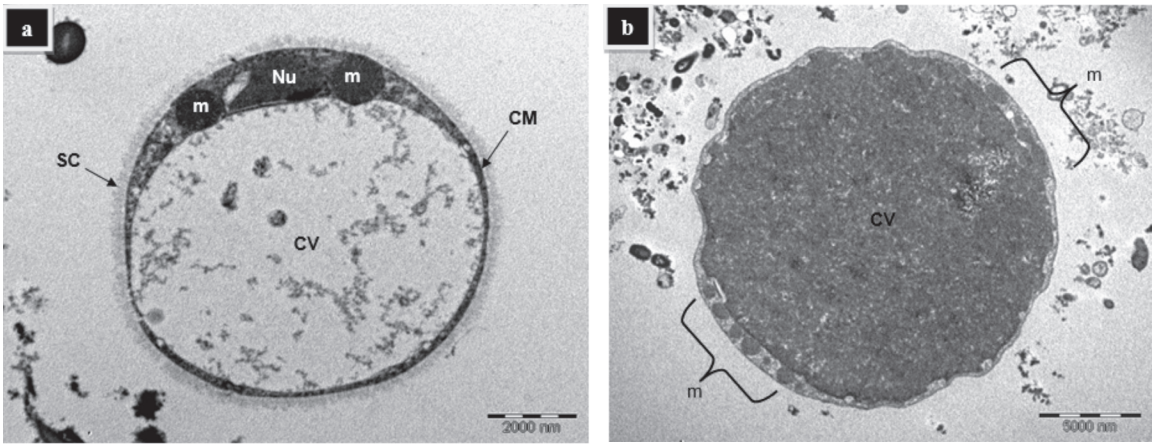

Fig 3 - Transmission electron micrographs showing *Blastocystis* isolated from (a) free-range chicken and (b) barn-reared chicken. CV, central vacuole; m, mitochondria; Nu, nucleus; SC, surface coat; CM, cell membrane.

DNA sequencing revealed. Four *Blastocystis* subtypes: ST1 was isolated from a free-range chicken. ST6 was isolated from two barn-reared chickens. ST8 was isolated from two free-range chickens. ST7 was isolated from four free-range chickens and one barn-reared chicken.

DISCUSSION

There are several published studies of *Blastocystis* isolated from birds worldwide (Table 1). Chandrasekaran *et al* (2014) reported high infection rates among ostriches in Malaysia. In this study, we found high *Blastocystis* infection rates among studied chickens in Malaysia especially in free-range chickens.

There are no previous reports of *Blastocystis* in barn-reared commercially bred Cobbs and Ross broiler chickens in Malaysia. A study by Lee and Stenzel (1999) found no *Blastocystis* infection among barn-reared chickens in Brisbane regional area of Australia due to good and proper farm management. However, in this study 11 out of 74 chickens sampled had *Blastocystis*. It is suggested that poor hygiene practices and lack of inspection in farms may contribute to *Blastocystis* infection in the barn-reared chickens.

Blastocystis is polymorphic and exists in many forms. In our study, the vacuolar form was the most common form seen. *Blastocystis* sp isolated in our study were morphologically similar to those isolated from humans (Matsumoto *et al*, 1987). However, in our study, *Blastocystis* sp had large variations in size similar to a study by Yamada *et al* (1987). The large cell bodies of *Blastocystis* isolates in our study from the barn-raised chickens had vacuolar forms containing electron-dense material when seen under transmission electron microscope. Sudan staining revealed that electron-dense material was due to lipid accumulation similar to the findings of Chandrasekaran *et al* (2014) in a study among ostriches. The lipid storage seen in the vacuoles of *Blastocystis* isolates could be due to the poultry diets which contains high-fat pellets.

Table 1
List of previous publication on *Blastocystis* in chickens.

Host	Reference/ Origin	Prevalence (%)	Morphologica characteristics			Genotypes
			Light microscope	SEM	TEM	
Chicken	Yamada *et al.* (1987)/ Japan	- 10/10 (100)	- Although polymorphic forms were rarely found in the lumen of the chicken's caecum, these forms were frequently found shortly after *in vitro* cultivation. - The central vacuole usually did not stain with iodine, but was sometimes lightly to heavily stain. - Size: 9-32 μm in the lumen contents of fowl caeca.	-	-	-
	Belova and Kostenko (1990) : Hen	- 80 - 100%	- The parasite form varies from round to ellipsoid. - There were 1 to 4 nuclei - Cytoplasm contains a great number of ribosomes and mitochondria with cristae resembling in their shape oval or round small sacs.	-	-	-

Table 1 (Continued).

Host	Reference/ Origin	Prevalence (%)	Morphologica characteristics			Genotypes
			Light microscope	SEM	TEM	
			- Nucleus contains nucleolus. Chromatin mass is concentrated on one of the poles of the nucleus as individual bodies. - Sizes: 7.5-35.0x6.25-30.0 (18.67x17.05) μm.			
Chicken	Stenzel *et al.* (1994)/ Australia	- 14/18 (77.8)	- The cells were always large, irregularly shaped cells. - Size: 15-20 μm in diameter. Larger cells (>30 μm in diameter)	- The cells were surrounded by a thick, fibrillar surface coat.	- Vacuolar contents were varied, ranging from a few clumps of flocculent material to homogeneous electron-opaque contents. - Clumps of condensed chromatin were present in the nucleus. - The nuclei were more elongated. Multinuclated cells were frequently seen.	-
	Lee and Stenzel (1999)	- 215/227 (95)	- *Blastocystis* cells showed considerable variation in size, ranging from approx. 3 μm to approximately 120 μm in diameter.		- Nuclei showed "spots" of electron-opaque material, generally arranged as a band within the nuclei. - Multiple individual cysts within a single outer fibrillar layer were found in addition to single cysts without an encompassing fibrillar layer.	-

Table 1 (Continued).

Host	Reference/ Origin	Prevalance (%)	Morphologica characteristics			Genotypes
			Light microscope	SEM	TEM	
	Yoshikawa *et al*. (1996)/ Japan	-	-	-	-	ST1
	Yoshikawa *et al*. (2003)/ Japan	-	-	-	-	ST1 and ST6
	Arisue *et al*. (2003)/ Japan	-	-		-	ST6
	Noël *et al*. (2003)/ France	-	-		-	ST7
Chicken	Abe *et al*. (2004)/ Japan	-3/6 (50)	-	-	-	ST1 and ST7
	Alfellani *et al*. (2013)/ Libya	-1/3 (33.3)	-	-	-	ST7
	Bergamo do Bomfim and Machado do Couto (2013)/ Brazil	- 23/70 (32.9)	<u>Vacuolar</u> - The cells were rounded in shape and contain a central body resembling a large vacuole. <u>Granular</u> - The granular form showed a different quantity of granules in their interior. - Size: 9.0 to 28.3 μm.	-	-	-

Table 1 (Continued).

Host	Reference/ Origin	Prevalance (%)	Morphologica characteristics			Genotypes
			Light microscope	SEM	TEM	
			Amoeboid - It was found in small a-mounts in the stained fecal smears. - Size: 13.4 to 45.5 μm. Cyst - The cells were characterized as rounded or ovoid, with one or two internal nuclei. - Size: 2.1 to 5.5 μm.			

A previous study (Abe *et al,* 2003) reported finding multiple subtypes of *Blastocystis* in chickens, namely ST1, ST2, ST4, ST6, ST7 and ST8. In our study we identified ST1, 6, 7 and 8. ST6 was identified from two barn-raised chickens. This subtype was also found in a Chinese bamboo partridge, a vulturine guineafowl and a Japanese green pheasant at a zoo in Japan (Abe *et al*, 2003). Other than birds, this subtype was also found in street dogs from India (Wang *et al*, 2013). However, it is uncommon in humans with a prevalence of 1% in the Netherlands (Bart *et al*, 2013) and 3.6% in Thailand (Jantermtor *et al*, 2013).

ST7 was previously reported in animals in Japan: chicken (Yoshikawa *et al*, 2003), quails (Yoshikawa *et al*, 2003; Abe 2004) and guinea fowl (Abe *et al*, 2003) as well as in ducks from France (Noël *et al*, 2003). Besides, ST7 has also been reported to infect humans and goats from Malaysia (Tan *et al*, 2013). In our study, ST7 was found in isolates from free-range chickens and barn-reared chicken. Therefore, it is suggested that domestic animals such as goats and chickens may serve as reservoir for humans infection in Malaysia.

ST8 is commonly found in non-human primates but rarely been reported in humans. However, Stensvold *et al* (2009) found this subtype in woolly monkeys and their handlers in the United Kingdom. A study by Samseh Abdullah *et al* (2017) conducted in the Orang Asli (aborigines) settlements in Temerloh, Pahang, Malaysia detected ST8 in goat and dogs but none in chickens. In our study, ST8 was found in two free-range chickens.

ST1 was found in free-range chickens in our study and has been previously reported in chicken in Japan (Yoshikawa *et al*, 1996), dogs in India and Brisbane,

Australia (Wang *et al*, 2013), swine in Spain (Navarro *et al*, 2008), chimpanzee and gorilla in New South Wales, Australia (Roberts *et al*, 2013). It is the most common subtype found in humans which has pathogenic potential (Moosavi *et al*, 2012). A study from Thailand reported 80% of school-going children examined were infected with ST1 (Jantermtor *et al*, 2013).

Blastocystis sp have little host specificity and increased risk for poultry to human transmission, especially among farmers, slaughter house workers, wet market workers and through consumption of contaminated meat, eggs and vegetables, since chicken manure is widely used as organic fertilizer for growing vegetables in Malaysia.

In conclusion, in our study, we determined the prevalence, subtypes, surface structure and ultrastructural of *Blastocystis* sp from chickens in Malaysia. These findings highlight zoonotic implication to humans especially among animal handlers in the chicken farming community. This study also recorded multiple *Blastocystis* subtypes in the chicken isolates.

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