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RESEARCH ARTICLE

Natural infection of *Blastocystis* ST6 among commercial quails (*Coturnix coturnix*) in Penang, Malaysia

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ABSTRACT

Blastocystis sp. is a unicellular, anaerobic intestinal protist regularly reported in humans and various animals worldwide. There seems to be little research on *Blastocystis* infection in poultry in Malaysia, and none on *Blastocystis* in quail specifically. In Malaysia, the consumption of quail meat and eggs is rapidly gaining popularity as a significant source of protein. It is, therefore, essential to explore the presence of *Blastocystis* in Malaysian quails in order to aid in the understanding of *Blastocystis* in this group of birds and their role in its transmission. Intestinal contents were collected from 90 commercial quails raised on two farms in Penang, Malaysia, in a multi-layer cage system with adequate farm management. Detection of *Blastocystis* sp. was by cultivation in modified Jones' medium supplemented with 10% horse serum. Giemsa-stained slides made from positive cultures were used for morphological studies whereas *Blastocystis* subtyping was conducted by using Polymerase Chain Reaction (PCR). A prevalence of 17.8% (16/90) was recorded for *Blastocystis* sp. in quail in this study. The most common forms detected in the *in vitro* culture medium were vacuolar and granular forms with cell diameters ranging from 9.09µm to 33.33µm. None of the quail birds screened had any visible gastrointestinal symptoms or signs. All successfully sequenced isolates were identified as *Blastocystis* sp. ST6, one of the potentially zoonotic subtypes of *Blastocystis*. This study posits that the quail birds may serve as reservoirs of zoonotic subtypes of *Blastocystis.* More studies are required to understand the source of *Blastocystis* infection to poultry under intensive care and the role of poultry animals in the transmission of *Blastocystis* to humans.

Keywords:

INTRODUCTION

Blastocystis is a single-celled, anaerobe (Yildiz *et al*., 2016), and is one of the most regularly reported intestinal parasites in humans and other animals worldwide (Valença-Barbosa *et al*., 2019). Animal hosts of *Blastocystis* include amphibians, birds, cattle, insects, monkeys, reptiles, rodents, and pigs (Ahmed & Karanis, 2018). *Blastocystis* is an atypical member of the stramenopile group of eukaryotes with its lack of flagella and surface tubular hairs (Yason & Tan, 2018). This protist has been found in both symptomatic and asymptomatic individuals, which makes its pathogenic role undetermined (Deng *et al*., 2021b). *Blastocystis* cells are spherical and have a large vacuole inside, with multiple other internal organelles in the peripheral rim that can be viewed when stained (Stensvold & Clark, 2016). There are four morphological forms mostly reported - vacuolar, granular, amoeboid, and cyst (Yildiz *et al*., 2016). Infection occurs by ingestion of cysts, and food and water borne transmission have been reported (Popruk *et al*., 2021).

Blastocystis has a high genetic diversity with at least 28 different subtypes (ST1-ST17, ST21, ST23-ST29 and ST30-ST32) identified based on polymorphism of small subunit ribosomal RNA (SSU rRNA) (Higuera *et al*., 2021; Maloney *et al*., 2021; Maloney & Santin, 2021). Frequently isolated subtypes from humans are *Blastocystis* sp. ST1 to ST9 and ST12 while all subtypes, except ST9, have often been documented for one or more animal groups (Alfellani *et al*., 2013a; Jiménez *et al*., 2019; Hublin *et al*., 2020; Higuera *et al*., 2021). However, there is a report of the isolation of ST9 from chickens in Malaysia (Noradilah *et al*., 2017) and from ring-tailed lemurs in China (Ma *et al*., 2020).

In Malaysia, the characteristic presence of ST5 in pigs, ST6 and ST7 in poultry (Noradilah *et al*., 2017; Farah Haziqah *et al*., 2018), ST10 and ST14 in goats (Noradilah *et al*., 2017; Kamaruddin *et al*., 2020), cattle (Mohammad *et al*., 2018a),

deer (Mohammad *et al*., 2018b) underscore suggestions that these STs are specific to the respective animal hosts. Moreover, very recently, Siti Alawiyah *et al*. (2021) isolated zoonotic subtype, ST7 (allele 99, 100, and 101) in turkey populations from Penang in which this ST7 with different alleles were also reported in patients with *C. difficile* infection (CDI) from Singapore (Deng *et al*., 2021b) suggesting that zoonotic transmission of subtypes common to humans and other animals is, thus, implied.

There are quite a number of reports on *Blastocystis* in domestic and wild birds (Hublin *et al*., 2020), with chickens and ostriches being the most studied. Only a few of these have documented the prevalence of *Blastocystis* in quails (Bergamo do Bomfim & Machado do Couto, 2013; Monte *et al*., 2018; Maloney *et al*., 2020). Quails were found to harbour a large number of endoparasites including *Blastocystis* (Monte *et al*., 2018). No studies have hitherto described the presence of *Blastocystis* in Malaysian quails, highlighting a research gap that should be investigated. More importantly, zoonotic subtypes of *Blastocystis* have been discovered from a variety of bird species (Hublin *et al*., 2020), and the usage of quail birds as farm and pet animals is becoming more popular in Malaysia. In this study, the presence of *Blastocystis* in Malaysian quails was investigated to aid in the understanding of *Blastocystis* in this group of birds and their role in its transmission.

MATERIALS AND METHODS

Ethical approval

The birds in this study were handled according to Animal Ethics Approval: USM/IAUC/2019/(116)(967). Permission for sampling activity was obtained from Department of Veterinary Services (DVS) Penang, Ministry of Agriculture and Agro-based Industry, Malaysia.

Sampling

Sampling was carried out in two local farms in Penang, Malaysia, where birds are kept in an intensive, multi-layer cage system with adequate farm management. A total of 90 individual quail birds involved in this study in which only a small amount of the intestinal contents obtained from the newly slaughtered quails were collected and placed into labelled sterile stool collection bottles and transported to the laboratory immediately.

In vitro cultivation

A pea-sized amount of the intestinal content of each bird was introduced into 3ml of modified Jones' medium supplemented with 10% heat-activated horse serum (Chandrasekaran *et al*., 2014). The sample was incubated vertically at 37°C for 24 to 36 hours before it was examined. A drop of the sediment was extracted from the tubes and was examined under the microscope at 400x for the presence of *Blastocystis* sp. *Blastocystis* positive cultures were sub-cultured for 2 to 3 days before they were stored at -20°C for molecular characterization. Samples were considered negative if *Blastocystis* sp. forms were absent after the subculturing for 2 to 3 days.

Giemsa staining

Wet smears were made from the sediments of *Blastocystis* positive cultures on clean, grease-free slides. The smears were allowed to dry and were fixed in methanol. These were then stained using a 10% Giemsa stain for 45 - 60 minutes. Stained slides were viewed under a light microscope using 1000x magnification. The sizes of the *Blastocystis* cells present were recorded.

Blastocystis subtyping and phylogenetic analysis

Genomic DNA was extracted from *Blastocystis* positive cultures using the Nucleospin DNA stool kit (Macherey-Nagel, German) following the manufacturer's instructions. Samples were then subjected to DNA barcoding according to the method of Mohd Zain *et al*. (2017). This involved amplification of *Blastocystis*-specific SSU rRNA in a single step PCR reaction using primers RD5 (52 -ATCTGGTTG ATCCTGCCAGT-32) and BhRDr (52 -GAGCTTTTTAACTGCAACAACG-32). PCR products were sent to Apical Scientific Sdn. Bhd. for purification and sequencing.

The SSU rDNA gene sequences obtained were queried into the National Centre for Biotechnology Information (NCBI) using the nucleotide Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/BLAST) for *Blastocystis* subtype identification; and the *Blastocystis* Sequence Typing website (https://pubmlst.org/*Blastocystis*/) for subtype confirmation and allele identification.

Phylogenetic analysis was carried out using the MEGA X software. Nucleotide sequences obtained from this study were aligned with nucleotide sequences of accepted *Blastocystis* subtypes from the GeneBank using the Muscle algorithm. A phylogenetic tree was constructed using the Maximum Likelihood method, based on evolutionary distances calculated by the Tamura 3-parameter model. The tree was rooted using *Proteromonas lacertae* as an outgroup, and a total of 118 positions were included in the final dataset that included 28 nucleotide sequences. Bootstrapping with 1,000 replicates was used to determine support for the clades generated.

RESULTS

A total of 90 quail birds were examined for the presence of *Blastocystis* by *in vitro* cultivation. This study revealed that there was relatively low occurrence of *Blastocystis* in the quail samples with a prevalence rate of 17.8% (16/90) samples. The vacuolar and granular morphological forms were the most common in *in vitro* cultivation. *Blastocystis* isolates of quail ranged in diameters from 9.09µm to 33.33µm with an average diameter of 16.76µm (Figure 1). None of the birds screened in this study had any noticeable signs of lesion in the gastrointestinal tracts or other gastrointestinal symptoms or signs.

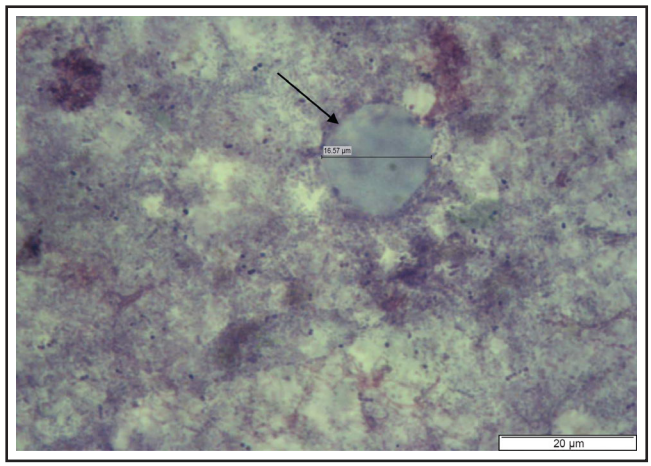

Figure 1. Vacuolar form of *Blastocystis* isolated from quail recovered from *in vitro* culture (arrow).

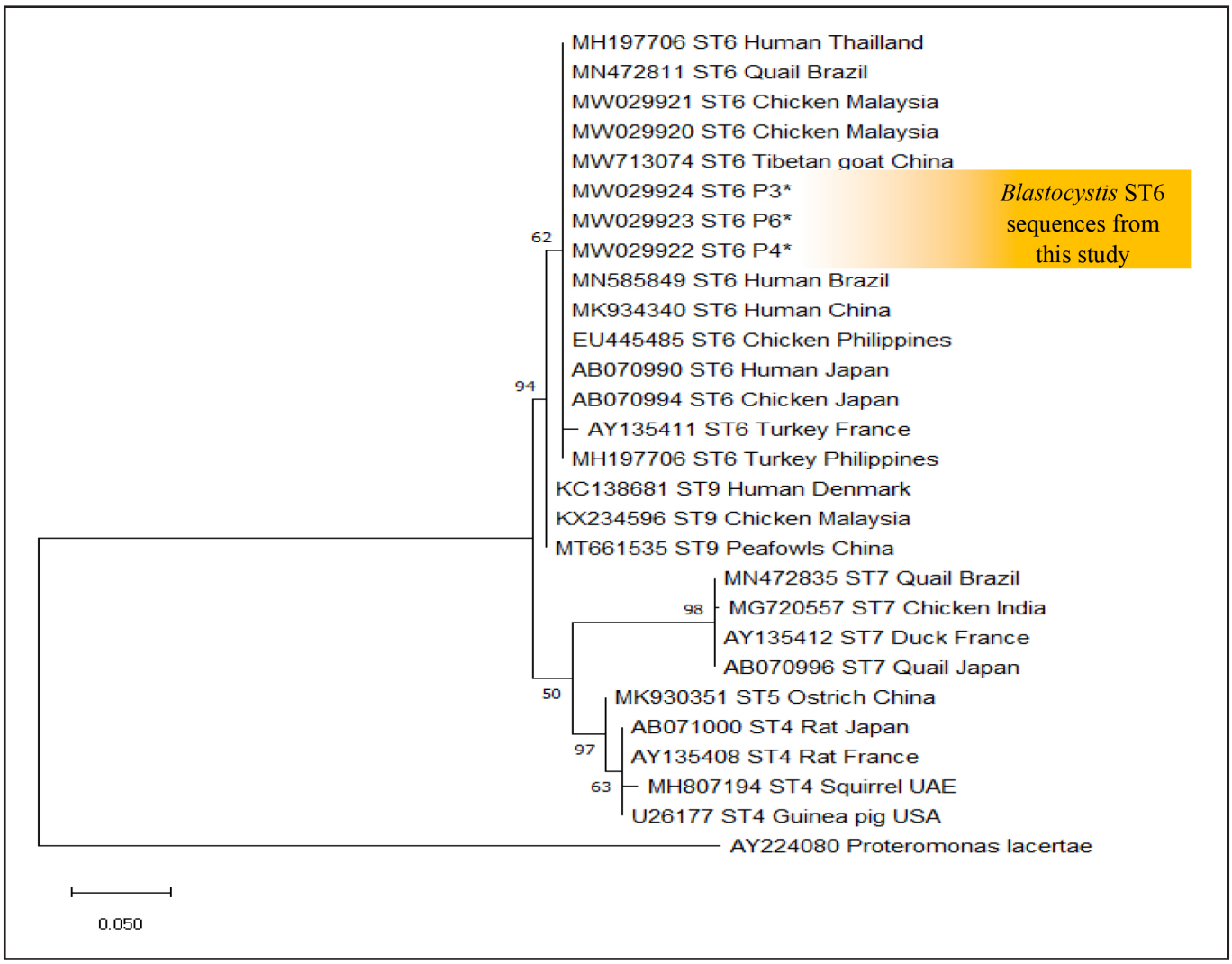

Figure 2. Phylogenetic relationships among *Blastocystis* sp. SSU rRNA gene sequences. The evolutionary tree was constructed using the Maximum Likelihood method, based on evolutionary distances calculated by the Tamura 3-parameter model (MEGA X software). The numbers on internal branches are percentage bootstrap values of 1000 replicates. The scale bar indicates an evolutionary distance of 0.05 nucleotides per position in the sequence. Sequences were represented by GenBank accession number, subtype, host, and country. *Blastocystis* sp. subtypes identified in this study were indicated by an asterisk after the identification number. *Proteromonas lacertae* was used as an outgroup.

Only three of the positive samples were successfully sequenced, all of which were identified to belong to *Blastocystis* ST6 (allele 122). The SSU rDNA gene sequences obtained in this study were deposited in the GenBank having been assigned the following accession numbers: MW029922, MW029923, and MW029924. The phylogenetic tree (Figure 2) showed that sequences from this study clustered with other *Blastocystis* ST6 sequences isolated from humans and other animals in several studies as obtained from the GenBank in which *Blastocystis* ST6 sequences from this study were closely related with the isolate from Tibetan goat from China (MW13074) and human isolate from Brazil (MN585849) with the bootstrap value 94.

DISCUSSION

Only a few studies have reported on *Blastocystis* in quail worldwide, mainly from Brazil and Japan (Yoshikawa *et al*., 2003a, 2003b, 2004; Arisue *et al*., 2003; Bergamo do Bomfim & Machado do Couto, 2013; Monte *et al*., 2018; Maloney *et al*., 2020). Nevertheless, none has been reported on *Blastocystis* sp. from quails in Malaysia. This study revealed a relatively low prevalence (17.8%) of *Blastocystis* in the quail samples. This is comparable to the prevalence of 19.3% (6/31), 20% (1/5), and 23.9% (17/71) from domestic quails from Brazil reported by Monte *et al*. (2018), Maloney *et al*. (2020), Bergamo do Bomfim and Machado do Couto (2013), respectively; although the records from Brazil seem to be slightly higher. This study posits that the quails were naturally infected with this protozoan parasite because they were totally kept in multi-layer cage system with proper farm management as they were reared commercially for the eggs and meat production. Lee and Stenzel (1999) demonstrated that a good hygiene practice and proper farm management would be beneficial in the prevention and control measure for parasitic infection including *Blastocystis*. Moreover, no signs or symptoms of gastrointestinal problems were observed in any of the birds studied. Results from this study corroborates the previous study by Greige *et al*. (2018) who demonstrated that poultry were generally infected by *Blastocystis* sp. and are thus the natural hosts of this protist.

Variation in sizes of *Blastocystis* sp. isolates has been observed in different hosts. Several morphological forms of *Blastocystis* sp. isolates of quail have been documented to

range in diameters from 3 - 15µm (Monte *et al*., 2018). Cell diameters ranging from 10 - 100µm has been reported previously in vacuolar forms in chickens from Malaysia (Farah Haziqah *et al*., 2018). Isolates from domestic birds were observed to range in diameters from 18.3 - 20.3µm (Bergamo do Bomfim & Machado do Couto, 2013). Cell sizes of *Blastocystis* sp. isolates from varying host have been found to overlap, thus invalidating the use of size as a means of differentiating *Blastocystis* isolates.

The identification of *Blastocystis* ST6 from quails in this study is not surprising. *Blastocystis* ST6 and ST7 are the mainly reported subtypes in bird and are often referred to as avian subtypes (Alfellani *et al*., 2013b; Greige *et al*., 2018). Also, ST6 has been isolated from quails in Japan and Brazil (Yoshikawa *et al*., 2003a, 2003b, 2004; Maloney *et al*., 2020). Similarly, *Blastocystis* ST6 has been observed in humans in different parts of the world (Deng *et al*., 2021a; Popruk *et al*., 2021; Rauff-adedotun *et al*., 2021). Allele 122 of *Blastocystis* ST6 observed in this study has, in particular, been previously reported in several bird species in Colombia (Ramírez *et al*., 2014), in barn-reared chickens in Malaysia (Farah Haziqah *et al*., 2018); and in Colombian children (Ramírez *et al*., 2017) as well as from symptomatic patients in Iran (Rezaei Riabi *et al*., 2018). This suggests the possibility of transmission of this subtype. However, before now, Greige *et al*. (2018) had demonstrated that *Blastocystis* sp. ST6 is capable of zoonotic transmission through the identification of ST6 in both poultry animals and their in-contact slaughterhouse staff members in Lebanon. Besides, as indicated in the phylogenetic tree, *Blastocystis* ST6 sequences in this study were closely related to several human isolates particularly, from Brazil, China, Japan and Thailand which strongly indicate a high possibility of transmission of this subtype to human.

CONCLUSION

This study found that there was low prevalence of *Blastocystis* in quail populations in Penang, Malaysia. Surprisingly, it has generated a great deal of information on *Blastocystis* subtype isolated from quails namely, ST6 (allele 122). Based on the evolutionary relationship demonstrated in the phylogenetic tree, there is a high possibility of transmission of this subtype to human in which *Blastocystis* ST6 sequences in this study were closely related to several human isolates particularly, from Brazil, China, Japan and Thailand. Therefore, further examination on large sample size of quails including the surrounding environment especially the water source and their feed are needed in order to determine any possible source of contamination. Moreover, examination of animal handlers especially those who are in close contact with the birds are required as it may help to demonstrate evidently the zoonotic transmission of this ST or protozoa to Malaysians.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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