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RESEARCH ARTICLE

Molecular subtyping and phylogeny of *Blastocystis* sp. isolated from turkey (*Meleagris gallopavo*) populations in Penang, Malaysia

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ABSTRACT

Most poultry farms in Malaysia preferred rearing chickens either for eggs or/and meat than turkeys. This is due to several challenges such as parasitic load and heat stress in rearing turkey. *Blastocystis* is one of the most common protozoan parasites infecting poultry. As no study was conducted on *Blastocystis* infection in turkey in Malaysia, this study aims to determine the current status, the morphological characteristics and subtyping of *Blastocystis* from turkey reared either in closed house or free-range system in Penang, Malaysia. It was found that the prevalence of *Blastocystis* sp. infection in turkeys were moderately high with 41.6% (25/60) in the closed house and 45.0% (45/100) in free-range system as infection was higher in the female turkeys with no gastrointestinal signs and symptoms. Vacuolar form was the most common form found in the *in vitro* culture ranged between 5 to 20 µm in diameter with a rough surface coat and undulating cell surface viewed under the scanning electron microscope. Meanwhile, the ultrastructure of the cells from turkey isolates were varies with partially expanded electron-opaque vacuoles to electron-dense in fully distended vacuoles. Interestingly, sequence analysis for 30 positive *Blastocystis* isolates from turkeys revealed one subtypes with three alleles namely, ST7 allele 99 (73.4%, *n*=22), ST7 allele 100 (23.3%, *n*=7) and ST7 allele 101 (3.3%, *n*=1). Findings from this study added to our understanding on *Blastocystis* infection in turkey production.

Keywords: *Blastocystis*; Malaysia; Penang; protozoan; turkey.

INTRODUCTION

Wild turkeys are huge, sexually dimorphic fowls with long feet, wide and curved tails, elongated necks and small heads (Miller, 2018). Wild turkey (*Meleagris gallopavo*) is associated with the other members in the order Galliformes, family Meleagridae and genus *Meleagris*. Wild turkeys are very adjustable in various conditions, capable to live in warm environments as well as to some countries that are frequently blanketed with snow. The adult males, known as tom or gobblers, weigh from 10 to 15 kg throughout their range depends on the type of breeds. The adult females, known as hens, commonly do not surpass 10 kg, with the typical weight from 6 to 9 kg (Cathey *et al*., 2007). In Malaysia, turkeys are reared for many purposes such as poultry meat as well as a hobby. Turkeys are considered expensive and have a high demand especially during festive season such as Christmas Eve and Deepavali.

The turkey's usual behaviors are to forage food on soil, therefore, there are numerous types of organisms as well as intermediate hosts that can cause the endoparasites infection in turkeys as they are omnivorous, they have a wide-ranging diet. Mohammad Zarith *et al*. (2017) stated that studies on the dispersion of parasitic infection in turkeys particularly in Malaysia is still scarce which probably due to Malaysian preference to eat more chicken than turkey, making study on turkey diseases economically insignificant.

Generally, turkeys are having some issues to several parasitic diseases caused by protozoan parasites. Protozoa are single-celled organisms that can be commensals or parasitic in nature. There are certain species of parasitic protozoan which include in the medical importance worldwide. In turkey population, the most common species of parasitic protozoan encountered were *Eimeria* spp. which cause coccidiosis (Sharman *et al*., 2010) and *Histomonas meleagridis*, the source of Blackhead disease (histosomiasis). Other protozoan which may also infect turkeys include *Hexamita meleagridis* (Hexamitiasis), *Trichomonas gallinae* (trichomoniasis) and *Cochlosoma anatis* (cochlosomiasis) (Hauck & Hafez, 2012). Apart from that, a neglected zoonotic protozoan known as *Blastocystis* sp. was also been found in

turkeys (Lee, 1970; Yamada *et al*., 1987; Belova & Kostenko, 1990; Belova, 1992a; Mokhtar & Youssef, 2018).

Blastocystis sp. is a common, non-flagellated, anaerobic stramenopiles (Gentekaki *et al*., 2017) that inhabits the gastrointestinal tracts in many humans and various animals particularly poultry (Mokhtar & Youssef, 2018). *Blastocystis* exists in four different morphological form namely, vacuolar, granular, amoeboid and cyst form (Tan, 2008). The most common mode of reproduction is binary fission (Adao & Rivera, 2018) in which cyst is the infective form that accountable in the transmission. The main transmission mode of this protozoan is through the faecal-oral pathway via drinking untreated water and/or poor sanitary conditions.

The occurrence of this organism has been perceived in a wide diversity of species worldwide. It has a great genetic diversity thus the genotypes were assigned using the subtyping nomenclature (ST) (Rauff-Adedotun *et al*., 2020). Nomenclature *Blastocystis* sp. subtypes (STs) ST1-ST9 was first presented in 2007 (Rauff-Adedotun *et al*., 2020), after many of subtypes were proposed recently. Starting from the year 2013, new subtypes was recognized which was ST1-ST17 between some hosts (Alfellani *et al*., 2013; Stensvold & Clark, 2020). Presently, a total of 29 subtypes have been suggested (Rauff-Adedotun *et al*., 2020). However, four subtypes out of 29 subtypes that have been proposed namely, ST18, ST19, ST20 and ST22 was recently under question due to the probability that they were generated from memento consequently their quixotic emergence (Stensvold & Clark, 2020). The enduring 25 subtypes which include ST1-ST17, ST21, ST23-ST29 have encountered the existing suggested standards for distinctive subtype nominations (Maloney & Santin, 2021). Additionally, ten subtypes, ST1-ST9 and ST12 have been revealed in humans, with fluctuating stages of existence (Greige *et al*., 2019) later the possibility of zoonotic transmission will occur (Clark *et al*., 2013; Stensvold *et al*., 2020).

The most recent study on *Blastocystis* in poultry by Greige *et al.* (2018) reported that the avian samples specifically from chickens in Lebanon were subtyped and fitted to any ST6 or ST7, with a great majority belongs to ST6. Surprisingly, this subtype also been detected among the chicken handlers which affirmed that there was zoonotic transmission of this ST as those individuals were frequently in a direct contact with the chickens. Meanwhile, Mokhtar & Youssef (2018) reported the occurrence of ST1, the zoonotic subtypes with a prevalence of 7.8% in poultry species among the chicken, ducks, geese and turkeys isolates in Egypt. It was also been found in humans having similar ST with the animals that they handle. Besides, the study also reported the occurrence of ST7 and ST6 in both turkeys and chickens in which both subtypes were represented as avian-adapted STs.

Most of the previous studies on *Blastocystis* in poultry were concentrated on *Blastocystis* in domestic chickens (Stensvold *et al*., 2009; Alfellani *et al*., 2013; Ramirez *et al*., 2014; Greige *et al*., 2018; Mokhtar & Youssef, 2018; Wang *et al*., 2018; Deng *et al*., 2019; Rauff-Adedotun *et al*., 2020; Maloney *et al*., 2021), quails (Maloney *et al*., 2021), ducks (Maloney *et al*., 2020; Rauff-Adedotun *et al*., 2020; Fahim *et al*., 2021; Maloney *et al*., 2021) and ostriches (Chandrasekaran *et al*., 2014; Maloney *et al*., 2020; Rauff-Adedotun *et al*., 2020; Deng *et al*., 2021; Rudzinska *et al*., 2021; Zhang *et al*., 2021). As there are very limited study in turkey population worldwide (Lee, 1970; Belova, 1992a; Noel *et al*., 2003; Sreekumar *et al*., 2014; Mokhtar & Youssef, 2018; Maloney *et al*., 2020) and none was conducted in Malaysia, therefore, this study will help to provide a baseline study on this neglected zoonotic protozoan parasite infection in turkey population mainly in the northern region of Peninsular Malaysia.

MATERIALS AND METHODS

Ethical approval

All animals used in this study were handled according to Animal Ethics and USM Institutional Animal Care and Use Committee (USM IACUC), Universiti Sains Malaysia. Written permission was obtained from the authorities of Department of Veterinary Services as sampling activities were conducted in privately-owned and protected turkey farms.

Sampling sites

This study was conducted in the Seberang Perai, Penang (Latitude: 5.3700° N and Longitude: 100.4139° E) as almost 50% of farmers reared turkey in this area. Sites were chosen based on types of turkey rearing which was closed house system and free-range system. Sampling activities were conducted on a closed house located at Department of Veterinary Services Penang, Bukit Tengah and several selected backyard farms at Tasek Gelugor, Kubang Menerong and Kepala Batas, Penang.

Study population

By adopting convenience sampling method (Dornyei, 2007; Etikan *et al*., 2016), a total of 160 turkeys consisted of free-range and closed house reared turkeys which involved 90 males and 70 females were examined for *Blastocystis* sp.

The closed house turkeys comprised of commercial broilers that reared specifically for meat. In the closed house, the turkey's reared were the White Holland turkey. They were kept indoors, secluded, retained with controlled temperature and have a good ventilation. Besides, wood shavings were commonly used as deep litter or floor systems with slatted floor. The turkeys were reared by the integrated federal government authorities of Department of Veterinary Services Penang in which the adult female turkey sold to the farmers as an initiative programme from the government. There were 60 faecal turkey samples collected from the closed house involved 20 males and 40 females screened for *Blastocystis* sp.

The turkeys consisting of free-range turkeys were frequently seen in countryside locations where old-style poultry production was practiced. The turkeys reared breed namely, Black turkey and White Holland that were partially confined and allow to scavenge for food freely and return periodically to the homestead or barn for water and food sources such as kitchen waste or feed pallet. The turkeys were kept in a small barn with the build of fenced area to protect from the predator, especially in the night-time. In this study, 100 faecal turkey samples were collected from the sampling sites consists of 50 males and 50 females.

In vitro cultivation

A small amount of each faecal sample was inoculated into a sterile screw-top bottle containing 3 ml of modified Jones' medium supplemented with 10% heat-activated horse serum. Each sample was incubated vertically at 37°C for 24 to 48 hours. Later, a drop of the sediment was examined at 400x magnification for *Blastocystis* examination in which positive samples were those with the presence of *Blastocystis* sp. forms. Positive samples were subsequently maintained by sub-culturing every 2 to 3 days and were then stored at -20°C for molecular characterization.

Microscopy examination

Smears were carried out from day-3 positive culture samples. Later, these smears were fixed with methanol, stained with 10% Giemsa and then viewed under light microscope at 400x

and 1000x magnification for the meticulous observation of morphological characteristics.

Selected day-3 positive culture samples from closed house (B7c) and free-range (FM11) turkey were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). Phosphate buffered saline (PBS) pH 7.4 were used to wash the contents for three times. The samples were centrifuged for 5 minutes at 3000 rpm. 2.5% glutaraldehyde were used to fix the pelleted cells and post-fixed with 1% osmium tetroxide. Then, the specimens were mounted on polycarbonate membrane and dehydrated in increasing concentration of ethanol (30%, 50%, 70%, 90%). The specimens were critical-point dried with carbon dioxide coated with gold and viewed under a scanning electron microscope (SEM) at Centre for Global Archaeological Research, USM, Penang.

Meanwhile, for transmission electron microscopy sample preparation, phosphate buffered saline (PBS) pH 7.4 were used to wash the contents for three times, centrifuged for 5 minutes at 3000 rpm in which 2.5% glutaraldehyde were used to fixed the pelleted cells and post-fixed with 1% osmium tetroxide, pH 7.3 at 4°C, washed thoroughly with cacodylate buffer and post fixed for 30 minutes in 1% osmium tetroxide in cacodylate buffer. The fixed cells were dehydrated for 5 minutes in ascending series of ethanol (30%, 50%, 70%, 90%) and embedded in epoxy resin. Semithin sections were stained with toluidine blue. Ultrathin sections were cut using an ultramicrotome, contrasted with uranyl acetate and lead citrate and viewed under a transmission electron microscope (TEM) at Electron Microscopy Unit, USM, Penang.

DNA Extraction

Genomic DNA of *Blastocystis* sp. was extracted by using Nucleospin® DNA stool extraction kit (Macherey-Nagel, German) according to the manufacturer's protocol. The elution step was carried out using 100 µl instead of 200 µl in order to increase the concentration of total DNA.

DNA Barcoding

The *Blastocystis*-specific sequence of the primers used, BhRDr (GAGCTTTTTAACTGCAACAACG) and the broad-specificity eukaryotic-specific primer, RD5 (ATCTGGTTGATCCTGCCAGT) were used in a single step PCR reaction to amplify 600 bp region of rRNA (Clark, 1997).

Amplification of 2 µl genomic DNA was carried out in a 50 µl reaction containing 25 µl of master mix 1.0 µl of MgCl, and 0.5 µl of each primer. The thermal cycling parameters were comprised as follows; 30 cycles of 1 min respectively at 94°C, 59°C, and 72°C, with an added 2 min final extension (Thermal Cycler Bio-Rad, USA).

The amplification products were then electrophoresed in 1.5% agarose gels and Tris-Acetate-EDTA (TAE) buffer. Gels were stained with DNA gel stain and visualized using ultra-violet gel documentation system. The DNA fragment size was confirmed using a 100 base pair ladder. PCR products of approximately 600bp were sent to Apical Scientific Sdn. Bhd. for purification and sequencing.

The SSU rDNA sequences were then identified by BLAST analysis in the sequence database at Public Databases for Molecular Typing and Microbial Genome Diversity (PubMLST) (https://pubmlst.org/organisms/blastocystis-spp) sequences generated in this study were deposited in GenBank under the accession number MZ437439-MZ437977.

Sequence alignment and phylogenetic analyses

Nucleotide sequences were analysed using BioEdit version 7.2. Phylogenetic tree was then constructed with MEGA X Platform x86, x86-64 using neighbour joining p-distance model. The sequences isolate from this study together with other *Blastocystis* sequences from the GenBank.

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model (Tamura, 1992). The tree with the highest log likelihood (-11626.88) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura 3 parameter model, and then selecting the topology with superior log likelihood value. The phylogenetic tree was rooted using *Proteromonas lacertae* as an outgroup. This analysis involved 49 nucleotide sequences. There were a total of 1922 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018).

Statistical analysis

Statistical analyses were conducted by using the Statistical Package for the Social Sciences (SPSS) statistics 26.0 software package. Chi-square analysis was carried out to determine the statistical significance of the observed the association between the risk factors (sexes and rearing systems) and *Blastocystis* sp. infection with a probability value of less than 0.05 was considered statistically significant.

RESULTS

Prevalence of *Blastocystis* sp. infection

Out of the 160 turkeys, a total of 70 (43.8%) turkey faecal samples that were positive for *Blastocystis* sp. infection (Table 1) in which none of the study animals showed behavioural signs or indication of *Blastocystis* sp. infection.

The prevalence of *Blastocystis* sp. infection in turkeys reared in the closed house and free-range system were 41.6% (25/60) and 45% (45/100), respectively (Table 1). It was also found that there was no significant difference (P>0.05) reported between the type of turkey rearing system and *Blastocystis* sp. infection ($\chi 2$ = 0.169, [df] = 1, P= 0.681) in this study.

Meanwhile, the prevalence of *Blastocystis* sp. infection in turkey from closed house was higher in female with 60% (12/20) whereas in male with 32.5% (13/40). As, for the free-range turkeys, the prevalence was also reported higher in female with 56% (28/50) than in male with 34% (17/50) (Table 1). There was a significant difference (P<0.05) reported between the sex of turkeys and *Blastocystis* sp. infection ($\chi 2$ = 4.149, [df] = 1, P= 0.042) in this study.

Morphological forms

From the *in vitro* cultivation of *Blastocystis* sp. isolates in the turkey faecal sample, the morphology of the *Blastocystis* sp. obtained were mostly vacuolar form approximately from 5 to

Table 1. Prevalence of *Blastocystis* sp. infection in two types of farming practices in turkey population

Study animals	No. of faecal samples	No. of turkeys infected (%)
Closed-house turkeys		
Male	40	13 (32.5%)
Female	20	12 (60%)
Free-range turkeys		
Male	50	17 (34%)
Female	50	28 (56%)
Total	160	70 (43.8%)

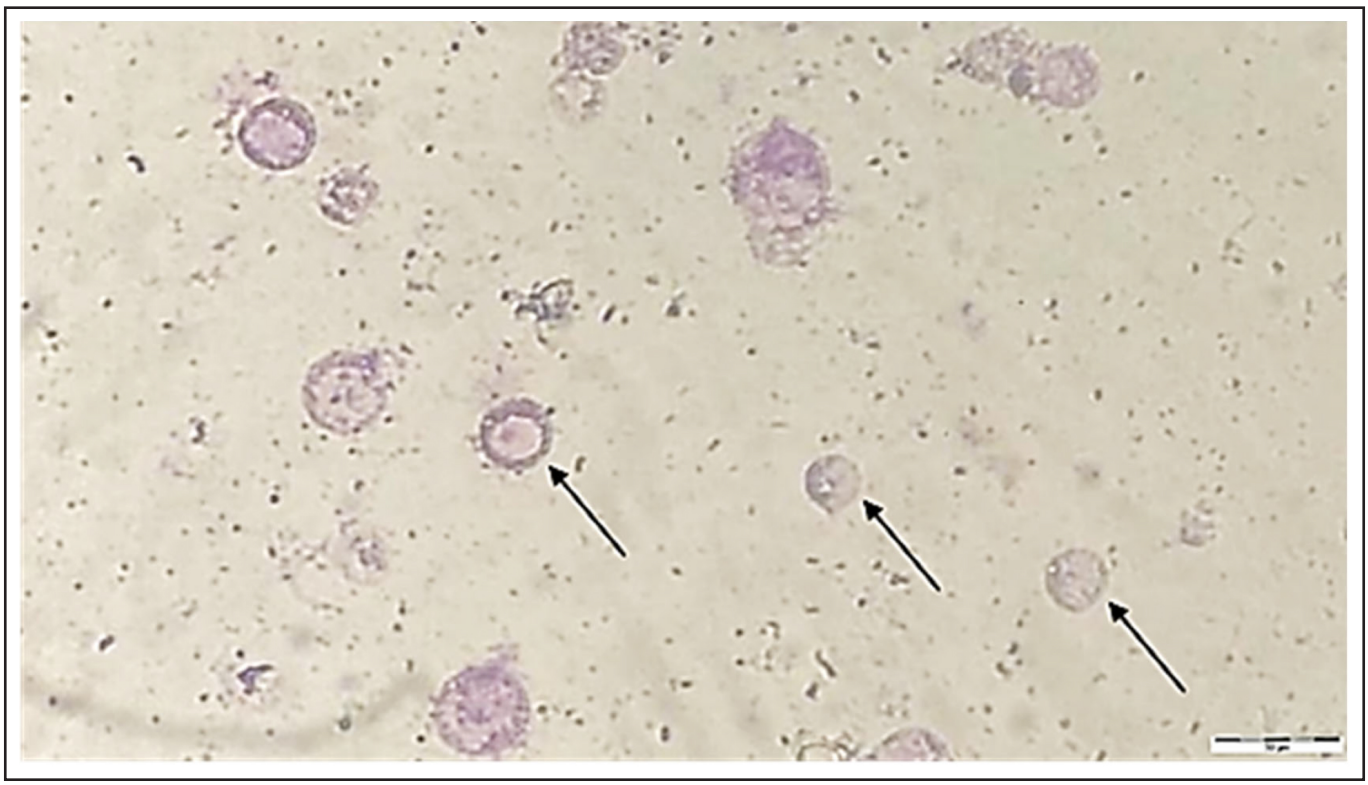

Figure 1. Vacuolar form of *Blastocystis* sp. in turkey (arrows).

20 µm in diameter (Figure 1). The granular forms size ranges from 5 to 30 ìm in diameter and it was commonly found in the older cultures of isolates (Figure 2).

Mode of reproduction

The morphology of the *Blastocystis* sp. and different modes of reproduction was observed under light microscopy in the *in vitro* cultures. Nevertheless, the mode of reproduction commonly observed in this study was binary fission (Figure 3).

Ultrastructure and surface structure

Scanning electron micrographs showed the surface structure of *Blastocystis* sp. isolated from the selected faecal culture of closed house (B7c) and free-range (FM11) turkeys. The cell surface for both isolates were generally spherical to rounded in shape and had a rough surface coat with undulating cell surface whereas some organisms showed gouges or deep furrows (Figure 4).

Blastocystis cell isolated from the selected faecal culture of closed house (B7c) and free-range (FM11) turkeys were examined by using transmission electron microscopic. It was revealed that *Blastocystis* cells from the close house turkey isolate showed a central vacuole with partially expanded electron-opaque vacuoles (Figure 5a) whereas *Blastocystis* cells from the free-range turkey isolate contained a large central vacuole with tiny electron-dense particles in fully distended vacuoles (Figure 5b). Besides, the organisms also possessed a thin wispy surface coat that resembles a slight ruffled appearance of the surface observed under the scanning electron microscope.

Subtype identification, alignment and phylogenetic analysis

According to the sequence analysis of 30 positive *Blastocystis* isolates, one genotypes and three allele were identified by BLAST queries at *Blastocystis* Sequence Typing Database (https://www.publmst.org/blastocystis): ST7 allele 99 (73.4%, *n*=22), ST7 allele 100 (23.3%, *n*=7) and ST7 allele 101 (3.3%, *n*=1).

In closed house rearing system, allele 99 was the most common allele found with the frequency of 60.0% (6/10), followed by allele 100 with 30.0% (3/10), and allele 101 with 10.0% (1/10). Meanwhile in free range rearing system, allele 99 was also the most common allele with the frequency of 80.0% (16/20), followed by allele 100 with 20.0% (4/20) and none was found for allele 101 in free range turkey rearing system (Figure 6).

Based on the allele distribution in sex of turkey, allele 99 was commonly found in male turkey with the frequency of 54.5% (12/15), followed by allele 100 with the frequency of 42.9% (3/15). In female turkey, it was found that allele 99 was the most common allele with the frequency of 57.1% (10/15), followed by allele 100 with 45.5% (4/15) and allele 101 with 10% (1/15).

The Maximum-likelihood (ML) phylogenetic tree was built to examine the positions of our new sequences against a selection of GenBank reference sequences. It was found that all the sequences obtained form a single clade as indicated in Figure 6.

DISCUSSION

In Malaysia, the broiler chicken, jungle fowl, village chicken and duck are available in numerous places as well as the cost is more affordable than the turkey meat. Turkey meat is typically sold at the average of RM25 to RM30 (Mohammad Zarith *et al*., 2017) per kg whereas chicken meat is approximately cost for about RM6 to RM10 per kg. In certain countries, market demand for turkey meat is less popular than chicken or even duck meat (Parrott & Walley, 2017). Turkey meat consumption is scarcer particularly in Malaysia rather than the western countries namely, Canada and United States

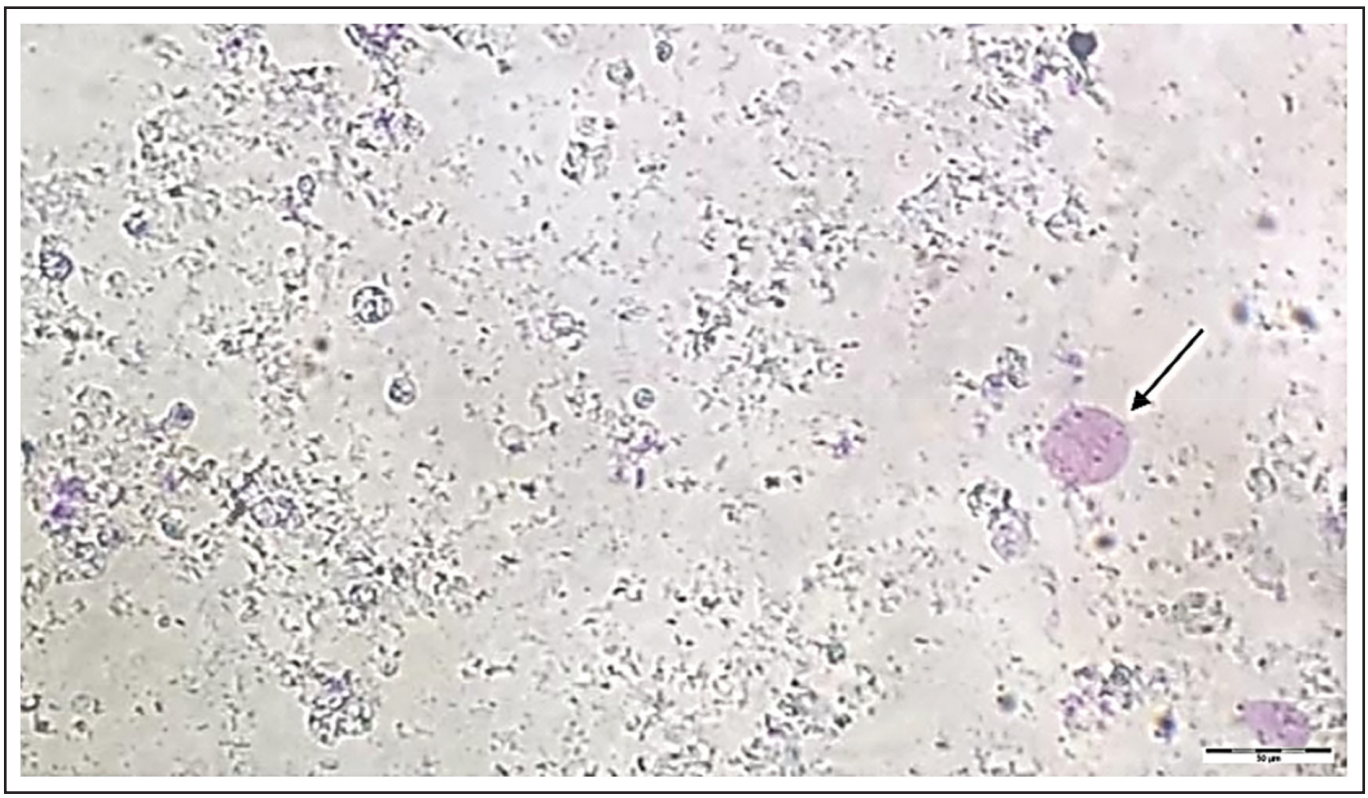

Figure 2. Granular form of *Blastocystis* sp. in turkey (arrow).

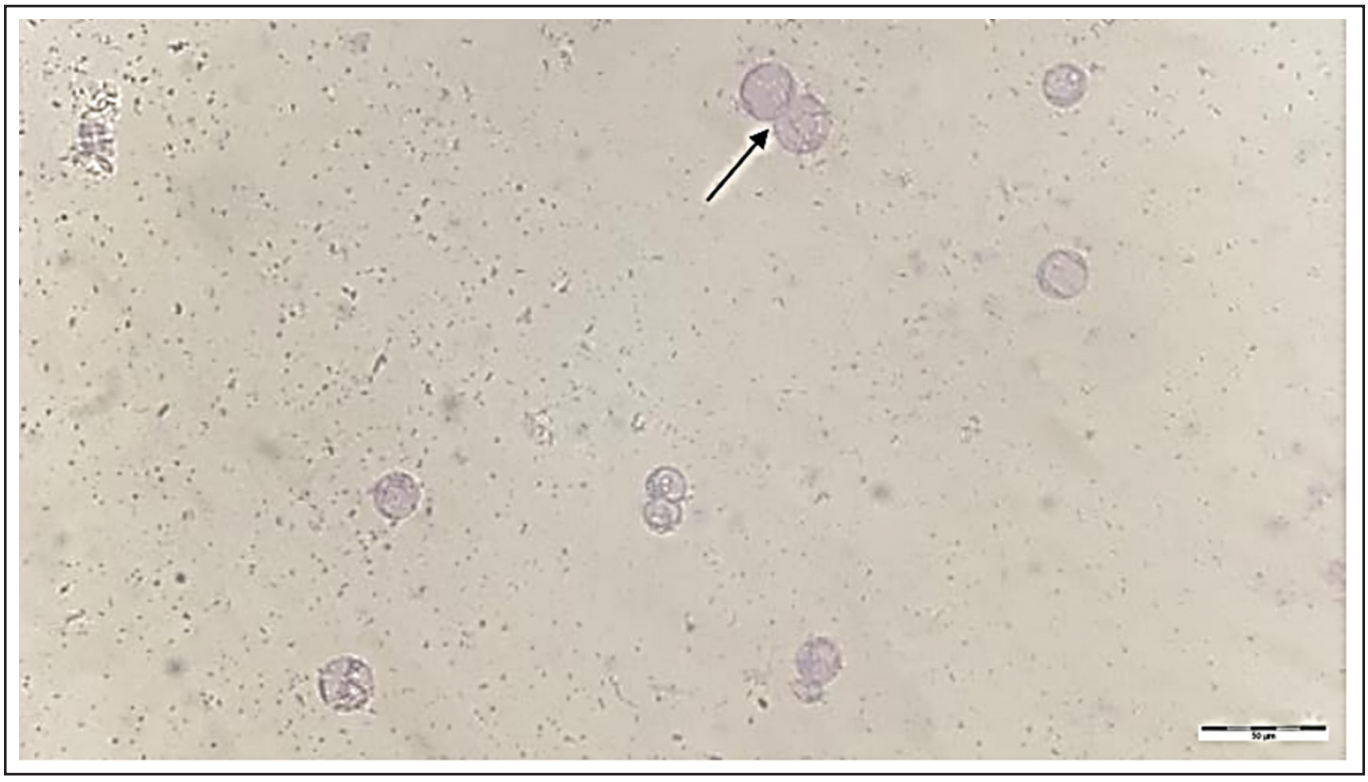

Figure 3. Binary fission, the reproduction mode of *Blastocystis* sp. (arrow) observed in turkey.

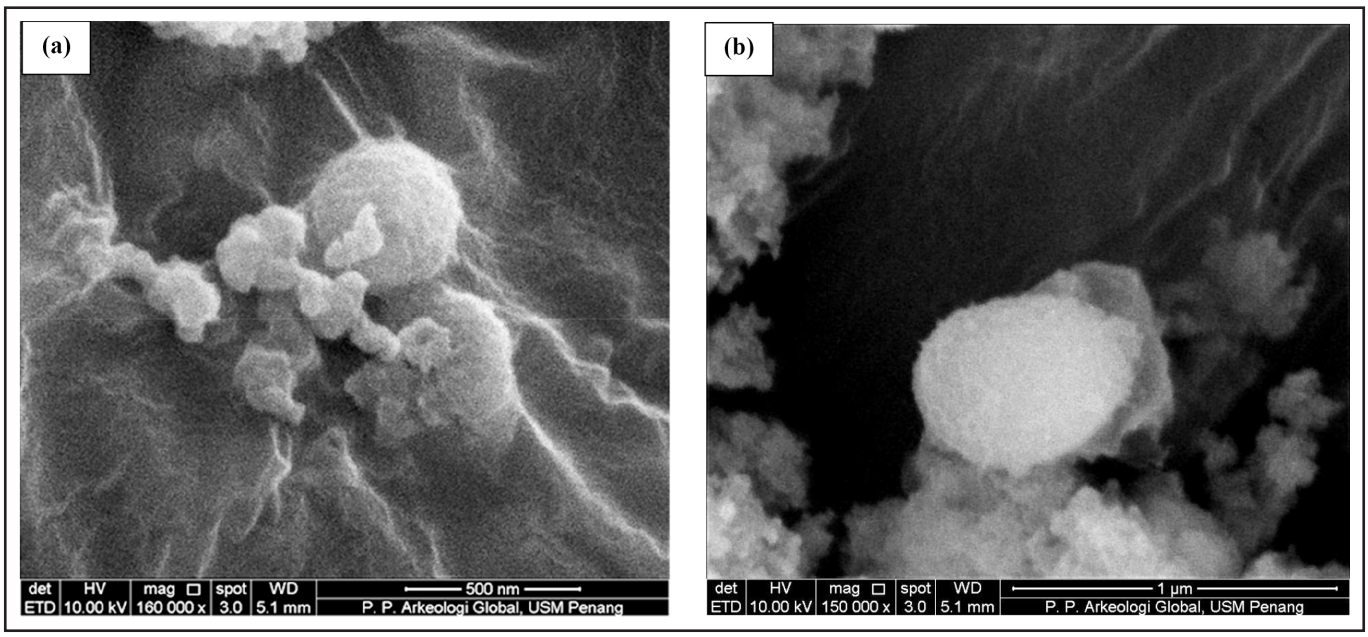

Figure 4. Surface structure of *Blastocystis* sp. (a) closed house turkey (B7c). (b) free-range turkey (FM11).

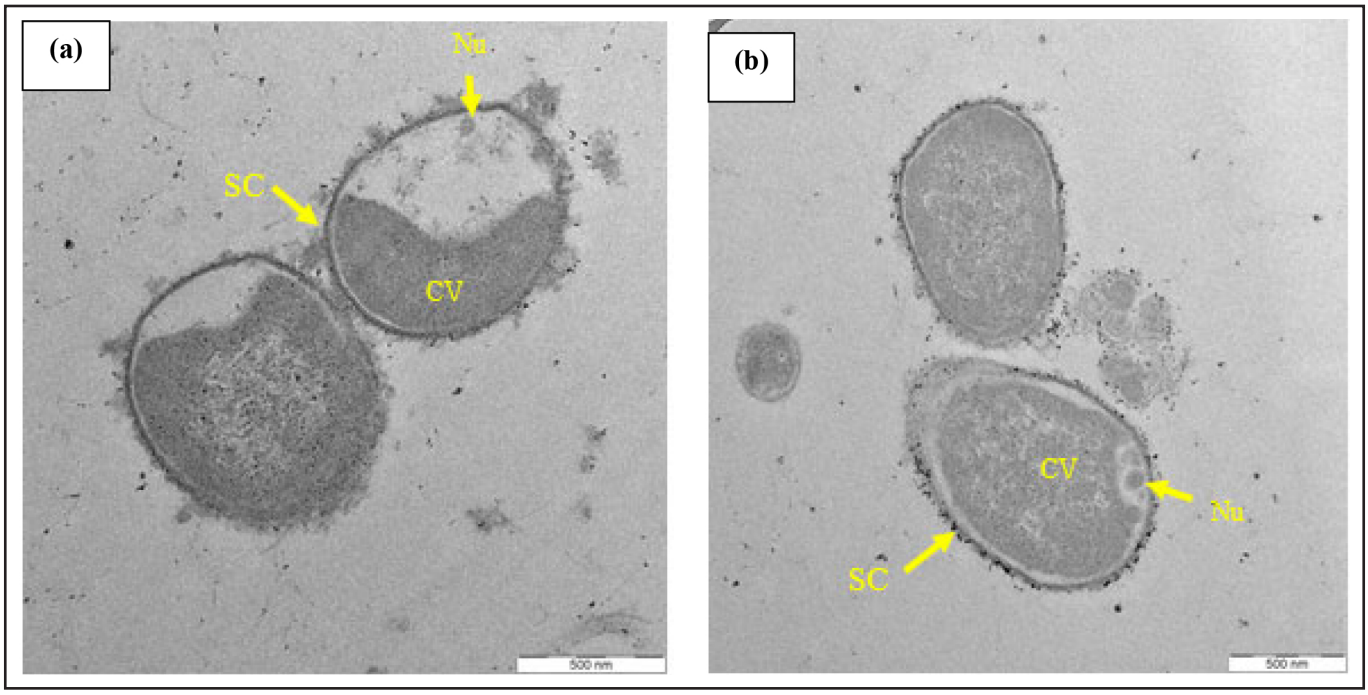

Figure 5. Transmission electron micrograph of *Blastocystis* sp. (a) closed house turkey (B7c) (b) free-range turkey (FM11). Nu; Nucleus, CV; Central Vacuole and SC; Surface Coat.

(Abduljaleel *et al*., 2012). Generally, cooking and eating turkey meat is associated with several festivities. In America and many parts of Europe, turkey meat will be served for dinner on Christmas Eve and the Thanksgiving Day. However, in Malaysia not only during Christmas Eve, turkey meat will also be being served during Diwali as a fascinating dish known as turkey biryani (Jayaraman *et al*., 2013).

Turkey population are not frequently been studied probably because they are less economically important to the poultry industry in Malaysia as compared to chicken and duck (Yadav *et al*., 2021). The only study on parasitic infection in turkey population in Malaysia was conducted by Mohammad Zarith *et al.* (2017) who reported on the occurrence of endo- and ectoparasites infection in free-range turkey population from Kedah, Malaysia. However, no attempt was made to detect the occurrence of the neglected zoonotic protozoan parasite, *Blastocystis* sp. infection in the turkey examined.

Studies on *Blastocystis* sp. infection was widespread and abundant in the animal population particularly in poultry, the avian population (Lee, 1970; Yamada *et al*., 1987; Belova & Kostenko, 1990; Pakandl & Pecka, 1992; Belova, 1992a, 1992b;

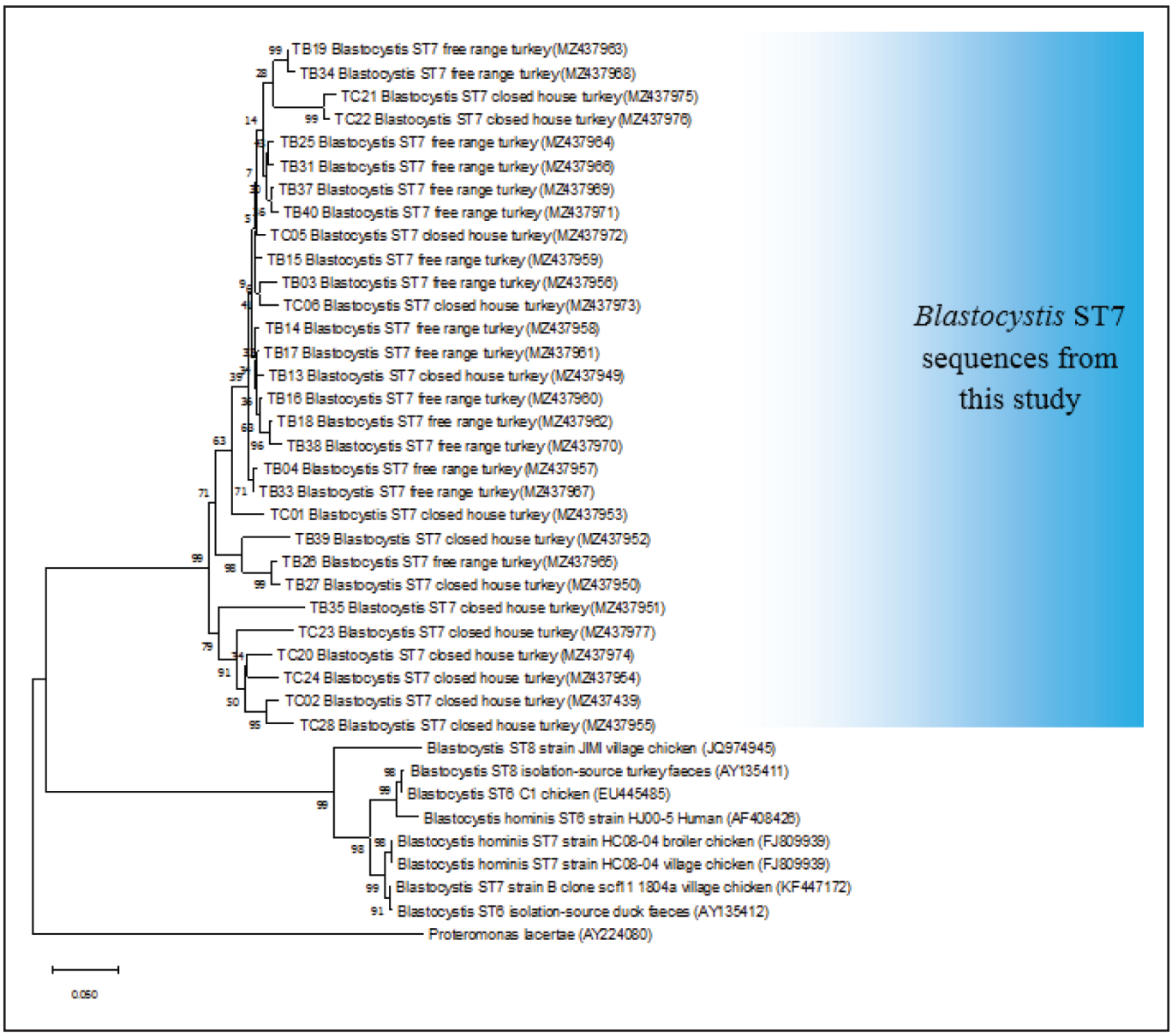

Figure 6. Phylogenetic tree of the new *Blastocystis* sp. sequences from turkey in Penang and reference SSU-rRNA gene sequences from GenBank.

Yoshikawa *et al*., 2003; Tanizaki *et al*., 2005; Stensvold *et al*., 2007; Tan, 2008; Clark *et al*., 2013; Adao & Rivera, 2018; Farah Haziqah *et al*., 2018; Greige *et al*., 2018; AbuOdeh *et al*., 2019; Mohammadpour *et al*., 2020; Oliveira-Arbex *et al*., 2020; Boutellis *et al*., 2021). Similarly, in Malaysia most studies were mainly focusing on *Blastocystis* infection in chicken population as they are the largest poultry production in the farming system in Malaysia (Farah Haziqah *et al*., 2018). To date, there are no known study on *Blastocystis* sp. infection in turkey *(Meleagris gallapavo)* population in Malaysia.

Therefore, this study was conducted to determine the current status of *Blastocystis* infection in turkey population in the Northern region in Peninsular Malaysia particularly in Penang, Malaysia as commercial turkey farming was currently increased at the mainland of Penang due to the support and assistance by the DVS Penang. It was found that the prevalence of *Blastocystis* sp. in turkey population was moderate with the prevalence of 43.8% (70/160) concurrent with *Blastocystis* infection in turkey from Egypt with 50% (6/12) prevalence (Mokhtar & Youssef, 2018). However, Sreekumar *et al.* (2014) reported high prevalence of infection in turkey from India with 70% (3/4).

Contrary to previous studies, this study examined a large number of turkeys with 160 animals were examined for *Blastocystis* infection with none of the positive turkeys showed behavioural signs or indication of infection. Apparently, other birds namely, ostriches infected with this protozoan parasite appeared healthy without any other symptoms as reported by Chandrasekaran *et al.* (2014). Currently, there is no conclusive evidence suggest the pathogenic role of *Blastocystis* infection in animals. However, *Blastocystis* may be a commensal organism that becomes pathogenic when the host is immunosuppressed, malnourished or has other source of infections such as bacterial or viral infection (Ginanjar *et al*., 2007; Lepczynska *et al*., 2016).

It was found that *Blastocystis* infection in free-range turkeys (45%) were slightly higher than the closed house turkeys (41.6%). Besides, based on the statistical analysis there was no significant different between different types

of rearing system and the infection of *Blastocystis.* Infection among free-range turkey flock was most probably due to the scavenging habits. Thus, the possibility of ingesting the infective stage of *Blastocystis* sp. in the environment appears to be very high due to the soil floor system in the backyard barns which makes them more susceptible to *Blastocystis* infection. Although, the closed house turkeys were totally confined in barren windowless enclosed long house with a deep litter system, *Blastocystis* infection was also reported to be relatively high in the closed house turkeys due to unhygienic practices in the pens such as the infrequent of changing the sawdust material of the floor system.

Despite the turkeys were reared under a supervision of a veterinary health officer and were treated with antibiotic and anthelminthic medication, both the closed house and free-range turkey population were found to be infected with a high prevalence of *Blastocystis.* Thus, excellent hygiene and sanitation are vital in avoiding or reducing the infection of *Blastocystis* because it is the main contributor for the health maintenance of poultry management (Stenzel & Boreham, 1996).

Finding from this study found that there was a significant difference between sex of turkey and *Blastocystis* infected. The prevalence of *Blastocystis* sp. infection in female turkeys were higher in both the free-range and closed house turkeys. The higher percentage of infection in the females may be due to the modification in the physiological condition of the animals during the production activity particularly during egg production in female turkeys as reported by Liu & Bacon (2005). Besides, Lioyd (1983) also reported that the advanced level of prolactin and progesterone hormones make the female ruminant more susceptible to any infection. In contrary to a previous study by Azhar *et al*. (2002), there was no variation in gastrointestinal parasitic infection between the sex of host. The faecal smears with vacuolar and granular forms were stained with Giemsa Stain for confirmative analysis. The size of the *Blastocystis* forms encountered varied from 5 to 20 µm. The measurements of the vacuolar forms of *Blastocystis* sp. in chicken were quite varied, with a minimum measurement of 10 µm and a maximum of 30 ìm in diameter (Farah Haziqah *et al*., 2014). According to difference in size and shape, the organism is occasionally difficult to identify by wet mount preparation. Conferring to the study of Zaki *et al.* (1991), enduring smears seem to be the technique of choice for light microscopic analysis. The staining characteristic of *Blastocystis* with Giemsa is alike to that defined by Yamada & Yoshikawa (2012) and Sreekumar *et al.* (2014) with the occurrence of an amorphous and granular substantial in the central vacuole and fluctuating number of nuclei (1-12) in the external rim of the cytoplasm. Morphological characteristics of *Blastocystis* in turkey isolates were observed. It was found that the most common form of *Blastocystis* in the in vitro culture was vacuolar. Moreover, granular form was commonly found in the older cultures of isolates.

Meanwhile, reproductive mode commonly observed in the *in vitro* culture of turkey faeces was binary fission which is characterised by the barrier of the cytoplasm of the mother cell and outcomes in two daughter cells with an identical size and shape. According to several studies on *Blastocystis* in poultry, there were two types of reproduction mode of the *Blastocystis* sp. been observed in poultry namely, binary fission and budding (Govind *et al.,* 2002; Yamada & Yoshikawa, 2012; Parija & Jeremiah, 2013; Farah Haziqah *et al*., 2014).

The surface structure for both isolates of closed house and free-range turkeys were generally spherical to rounded in shape and had a rough surface coat with undulating cell surface whereas some organisms showed gouges or deep furrows similarly indicated in the isolates from diarrhea cattle (Widisuputri *et al*., 2021). Meanwhile, Cassidy *et al.* (1994) revealed that the surface structure of chicken isolates appeared to be compact with a smooth and undulating cell surface. It is apparent that the surface structures of *Blastocystis* sp. from different hosts are variable, and this study notes the surface structure morphology in turkeys as none was reported previously in this bird. Moreover, surface coat may absent in certain forms namely, in the avacuolar form and the amoeboid form from human isolates as reported by Dunn *et al.* (1989) and Stenzel *et al.* (1991). It has also been suggested that the features of the surface structure of *Blastocystis* sp. maybe correlated with symptomatic appearance (Widisuputri *et al*., 2021).

Studies on the ultrastructure of *Blastocystis* sp. in poultry from Malaysia were previously reported in chickens (Farah Haziqah *et al*., 2018) and ostriches (Chandrasekaran *et al*., 2014). This study represented the ultrastructural features of *Blastocystis* vacuolar form isolated from the close house turkey isolate with a central vacuole contained partially expanded electron-opaque whereas *Blastocystis* cells from the free-range turkey isolate contained a large central vacuole with tiny electron-dense particles in fully distended vacuoles similarly reported in the barn-reared chicken (Farah Haziqah *et al*., 2018) and ostrich (Chandrasekaran *et al*., 2014) cells. Moreover, the ultrastructure features of *Blastocystis* in turkey was first demonstrated by Lee (1970) who also reported on the occurrence of finely granular material and crystalline inclusion in the central vacuole of the isolated *Blastocystis* cells. According to Yoshikawa *et al.* (1995), the dark electron-dense particles seen in the central vacuole indicating the presence of lipid. Therefore, it can be confirmed that *Blastocystis* sp. from turkey, chicken as well as the ostrich isolates uses the vacuolar forms to store lipids due to the poultry diets which contains high-fat pellets (Loar & Corzo, 2011; Evans *et al*., 2015).

There are very limited studies on subtype characterization of *Blastocystis* sp. isolated from turkeys (Noel *et al*., 2003; Mokhtar & Youssef, 2018). *Blastocystis* ST6 was reported in the turkey isolates from France (Noel *et al*., 2003) whereas variety of subtypes was isolated from turkey population in Egypt namely, ST1, ST6 and ST7 (Mokhtar & Youssef, 2018). *Blastocystis* ST1 was previously detected in variety of animal hosts namely, in chickens (Cian *et al*., 2017), dogs (Wang *et al*., 2013), pigs (Valenca-Barbosa *et al*., 2019), chimpanzees (Roberts *et al*., 2013), and gorillas (Roberts *et al*., 2013) as well as humans (Greige *et al*., 2018). Meanwhile, ST6 and ST7 were previously known as avian subtypes mainly because of its high prevalence in poultry specifically chickens, quails, geese as well as other bird population (Mokhtar & Youssef, 2018). However, ST6 and ST7 is scarce in humans with the prevalence as low as 1% infection of *Blastocystis* ST6 in the Netherlands (Bart *et al*., 2013), 3.6% infection of *Blastocystis* ST6 in Thailand (Jantermtor *et al*., 2013) and 1% infection of *Blastocystis* ST7 from the American continent (Jiménez *et al*., 2019).

From this study, ST7 was the only subtypes detected from 30 positive isolates with three different alleles namely, allele 99, 100 and 101. Interestingly, ST7 allele 99 was not only found in turkey, it was also been reported in other domestic animals namely, dogs (*n*=3) (Mohammadpour *et al*., 2020) and chicken (*n*=1) from Iran Rahimi *et al.* (2021). Notably, humans were also found to be infected with ST7 allele 99 as reported in a patient with Clostridium difficile infection (CDI) from Singapore (Deng *et al*., 2021) and one isolate from patient with diabetes mellitus in Brazil (Melo *et al*., 2020). Meanwhile, the only available data present to date for ST7 alleles 110 was by Lhotská *et al.* (2020) reported

the occurrence of this subtype in the gut-healthy humans from Czech Republic and Deng *et al.* (2021) reported in one of the patients with CDI from Singapore. As for ST7 allele 101, one isolate was reported from patients with CDI Deng *et al.* (2021) with 100% identity to those in humans in the Czech Republic (Lhotská *et al*., 2020). Since these subtypes were previously reported in human populations, animals may serve as reservoir hosts and facilitate transmission to human. Therefore, it can be suggested that transmission may occur between domestic animals to animals or humans.

In this Maximum-likelihood phylogenetic tree, the sequence of *Blastocystis* generated from this study form a well-supported because Bootstrap proportion and a long branch propagation to monophyletic group. This clade is the sister group of all GenBank sequence. There are low inter-sequences within this clade variability because sequences are very similar to one another due to same origin of the species. However, these sequences are quite distinct from their sister group sequences.

CONCLUSION

In this study, it was found that despite being raised in an intensive closed house system, treated with antibiotic and anthelminthic medication under a supervision of veterinary health officer, high prevalence of *Blastocystis* infection was observed in the closed house turkey population. It can be concluded that establishment with high-quality hygiene and sanitary conditions might result in negative infection as good hygiene practices will contribute to better health maintenance of the birds. Although, these studies have assisted in understanding the morphological characterization of this protozoan parasite in turkey, the morphological characteristics were in accord with the general features of *Blastocystis* in other bird hosts namely, chickens. Besides, this study has generated a great deal of data on subtype of *Blastocystis* isolated from turkeys in which zoonotic subtype, ST7 (allele 99, 100 and 101) were identified out of 30 positives isolates from turkey. Thus, zoonotic transmission should be taken into consideration as the animal handlers particularly, turkey farmers or the slaughter workers might have high risk of infection as they are in constant contact with the birds and more susceptible to *Blastocystis* sp. infection. To date, there is no information on *Blastocystis* infection in turkey population Malaysia, thus the findings of this study added to our understanding on *Blastocystis* infection as this is the first study to evaluate the current status, morphology, ultrastructure and genetic characteristics of *Blastocystis* sp. isolated from free-range and close house turkeys in Malaysia.

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Conflict of interest

The authors declare that they have no conflict of interest.

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