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RESEARCH ARTICLE

Epidemiological risk factors and phylogenetic affinities of *Sarcocystis* infecting village chickens and pigs in Peninsular Malaysia

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ABSTRACT

The intake of food and water containing the *Sarcocystis* parasite has been linked to a number of outbreaks worldwide, including Malaysia. Nevertheless, the lack of surveys and epidemiological data on *Sarcocystis* infections in Malaysia makes it difficult to estimate its occurrence in humans and animals. A cross-sectional study was conducted to determine the prevalence of *Sarcocystis* and the risk factors associated with infection among village chickens and pigs reared under different farm managements in Peninsular Malaysia. Phylogenetic trees were constructed using partial fragments of the 18S rRNA gene and ITS1 sequences. In the present study, 680 sera samples were collected from village chickens (*n*=250) and commercial pigs (*n*=433) and anti-*Sarcocystis* antibodies were screened using the enzyme-linked immunosorbent assays (ELISA) kit. At the animal level, the prevalence of *Sarcocystis* was 9.2% (95% CI: 5.92-13.48) and at the farm level, it was 64.0% (95% CI: 42.52-82.03) in village chickens. The animal-level seroprevalence of *Sarcocystis* for pigs was 3.7% (95% CI: 2.13-5.93) and 36.8% (95% CI: 16.29-61.64) at the farm-level. Polymerase Chain Reaction (PCR) was conducted on meat samples from various parts of village chickens (*n*=250) consisting of brain, heart, lung, and pectoralis muscle tissues, and pork (*n*=121) consisting of intercostal muscle, diaphragm, and tongue. *Sarcocystis* DNA was detected in 6.4% (95% CI: 4.60-11.60) of village chicken samples but zero in pork samples. A total of 11 unique *Sarcocystis* haplotypes were isolated from these tissue samples. Multivariable logistic regression analysis of the putative risk factors showed a statistically significant association between *Sarcocystis* infection in pigs and uncovered storage of feed. Although no zoonotic *Sarcocystis* was isolated in this study, we reported the first discovery of *S. wenzeli* in Malaysia.

Keywords: *Sarcocystis*; village chicken; pig; risk factor; phylogenetic.

INTRODUCTION

Sarcocystis species are common and are among the most pervasive zoonotic protozoan parasites, making them important both medically and economically (Chen *et al.*, 2012; Muir *et al.*, 2020). Humans may be either definitive or intermediate hosts, exhibiting intestinal or muscular forms of the disease. Individuals with intestinal sarcocystosis exhibit symptoms such as diarrhoea, stomach pain, and nausea, while muscular sarcocystosis can cause fever, myalgia, bronchospasm (Rosenthal, 2021). Human sarcocystosis is mainly acquired through the consumption of raw and undercooked meat and is potentially a threat to public health (Zhu *et al*., 2023). Species that are of primary importance for their zoonotic potential are *S. hominis* and *S. heydorni*, which are acquired through consuming infected beef (Rubiola *et al.*, 2020), *S. suihominis* from infected pork (Gazzonis *et al.*, 2019), and *S. nesbitti* from contaminated food or water (Shahari *et al.*, 2016). While intestinal sarcocystosis is widely reported across the globe, muscular sarcocystosis is uncommon, with nearly 50% of cases reported being the result of outbreaks in Malaysia (Fayer *et al*., 2015; Esposito *et al*., 2017). There have been several local outbreaks among visitors who visited Tioman and Pangkor Islands, highlighting the emergence of endemic *S. nesbitti* infections in the country (Arness *et al*., 1999; AbuBakar *et al*., 2013; Esposito *et al*., 2017).

According to the Department of Statistics Malaysia, chicken and pork are widely consumed in Malaysia, with yearly per capita consumption of 46.1kg and 17.3kg, respectively (Department of Statistics Malaysia, 2021). Village chickens are becoming more and more popular because people believe they are healthier and safer than commercial broiler chickens. However, due to their feeding habits, village chickens are discovered to be predisposed to *Toxoplasma gondii* (Leong *et al*., 2023) and *Sarcocystis* cysts (Pan *et al*., 2020) in their tissue organs through accidental ingestion of oocysts in the environment. Recently, more research has focused on avian species such as chickens due to the reported severity of sarcocystosis infection resulting in neurological signs and high mortality (Pan *et al*., 2020; Wilson *et al.*, 2020). The three species, namely *S. horvathi*, *S. gallinarum* and *S. wenzeli* have only been

reported in chickens (Memmedov, 2010; Chen *et al*., 2012; Pan *et al*., 2020). Pigs are found to harbour macro- and microcyst parasites such as *Taenia solium*, *Trichinella spiralis*, *Echinococcus granulosus*, *Toxoplasma gondii* and *Sarcocystis* spp. in the form of cysts in their tissue (Djurković-Djaković *et al*., 2013; Fazly *et al*., 2013; Pankaj *et al*., 2022). Pigs infected with *Sarcocystis* may exhibit symptoms such as fever and anorexia, and may result in pigs' deaths in severe cases (Caspari *et al*., 2011). *Sarcocystis miescheriana*, *S. porcifelis* and *S. suihominis* species have been discovered to infect pigs (Coelho *et al.*, 2015; Khademvatan *et al.*, 2017). Pigs acquire *Sarcocystis* spp. by accidently ingesting environmentally resistant sporocysts (Tenter, 1995). Sarcocystosis in livestock is responsible for the reduced quality of meat and the condemnation of carcasses, leading to economic losses (Avapal *et al*., 2004).

Considering Malaysia is one of the main countries where human sarcocystosis has been reported, this study determines the prevalence of *Sarcocystis* among the native village chickens and pigs, and identifies the farm-level risk factors that may influence the seropositivity in these food animals. Phylogenetic analyses based on the 18S rRNA and ITS1 genes are performed in order to determine the evolutionary relationships of the *Sarcocystis* isolates.

MATERIALS AND METHODS

Study areas

The study was carried out in Peninsular Malaysia, which is at the southernmost point of the Asian continent. It is bordered by Thailand to the north and separated from Singapore to the south. Peninsular Malaysia experiences a hot tropical climate with an average temperature of 28.52°C and rainfall ranging from 17000 to 5800mm throughout the year (Malaysian Meteorology Department, 2020). This study was conducted in Penang (approx. 5° 17' N and 100° 27' E), Perak (approx. 4° 35' N and 101° 5' E), Selangor (approx. 3° 30' N and 101° 31' E), Melaka (approx. 2° 12' N and 102° 14' E), and Johor (approx. 1° 31' N and 103° 44' E) in Peninsular Malaysia. The map of the study area is presented as supplementary information (Figure S1).

Study design and sample size estimation

A cross-sectional study was carried out from February 2019 to September 2020. The calculation of sample size was performed using the OpenEpi (version 2.3) software, with an assumption of expected seroprevalence of 20% based on a preliminary study of another protozoan parasite from the same family (*Toxoplasma gondii*) in village chickens (Sabri *et al.*, 2019) due to no reports of *Sarcocystis* prevalence in chickens, and 50% for pig samples based on a sarcocystosis study in pigs (Zainalabidin *et al*., 2017). Based on the calculation, a total of 250 and 433 village chickens and pigs were included in this study for serological testing, respectively. For PCR detection, a total of 1000 samples from 250 village chickens and 121 tissue samples from pork were collected, respectively. According to data from the Department of Veterinary Services Malaysia (DVS, 2020), the states with the biggest populations of pigs and chickens were chosen for sampling. Permission for sample collection was given by the Research and Innovation Division, DVS as part of their ongoing annual disease surveillance program, and consent was obtained from randomly selected farms. The number of farms involved in this study was calculated under the supposition that *Sarcocystis* can be detected in at least 10% of the farms with a precision of 10% at a confidence level of 95% (Andreopoulou *et al.*, 2023), given the uncertain prevalence of infections at farm level. Flocks containing >10 chickens per farm between the ages of 6-12 months and herds containing >25 pigs per farm that are older than 5 months were eligible for inclusion in this study. Based on the calculation, a total of 10 village chickens and 25 pigs were examined from each farms.

Farm characteristics

The majority of village chicken farmers that were invited to participate in this study raised their chickens on small-scale farms and in rural areas with plenty of outdoor areas away from major roads. The chicken farms sampled in this study were divided into two management categories: free-range and intensive. In the free-range farm, the birds were allowed to roam and forage throughout the day but returned to the coop at dusk, and given kitchen leftovers. The intensively managed chickens were kept and fed in an enclosed outdoor pen and given limited freedom to roam.

The local pig farms were divided into the open-house and closed-house management system. The pigs in the open-house farms were maintained in open-sided sheltered pens with concrete flooring. The house was not adequately fenced, allowing easy access of stray animals to the pens. The farms implementing the closed-house system had high security measures in place to keep stray animals away from the farm, cooling pads for proper ventilation, and zero wastewater discharge into the public drainage system.

Sample collection

The protocol involving chicken slaughtering and blood collection from live pigs was approved by the Animal Care and Use Committee (ACUC) of Universiti Putra Malaysia via Animal Use Protocol (AUP) reference number UPM/IACUC/AUP-R033/2019 prior to sample collection. Ten chickens were bought from each farm in batches and 3 ml of blood were obtained from each bird upon slaughtering. Pigs were restrained manually and an 18-gauge needle fitted with a vacutainer tube was used to draw 3ml of blood from the jugular vein. These tubes were labelled and transported on ice to either the Parasitology Laboratory at the Faculty of Veterinary Medicine, Universiti Putra Malaysia, Selangor, or the regional veterinary laboratories of the DVS for processing.

Serological examination

Chicken and pig sera samples were centrifuged using a refrigerated Eppendorf centrifuge (Model: 5804 R, Eppendorf, USA) at 4000 g for 10 min. The sera were put into a 1.5ml microcentrifuge tube and stored at -20°C until the serological test. Antibody detection was carried out using indirect commercial enzyme-linked immunosorbent assay (ELISA): Chicken *Sarcocystis* (Sunlong Biotechnology, Shanghai, China) and Porcine *Sarcocystis* ELISA kits (Sunlong Biotechnology, Shanghai, China). According to the manufacturer, the values of sensitivity and specificity for the two kits were over 98.0%. Antibody detection tests were carried out according to the manufacturer's protocol.

Tissue samples collection

One thousand tissue samples consisting of the brain, heart, lung, and pectoralis muscle tissues were collected from 250 chickens, weighing approximately 100g each. Tissue samples from the same chicken were pooled, put in a clean plastic bag, and labelled. A total of 121 samples of pork were purchased and collected from butcher shops and meat kiosks. The intercostal muscle (n=68), diaphragm (n=17), and tongue (n=36) were obtained from the Klang Valley region of Selangor, Malaysia, and each weighed between 50 and 100g. Pig farms visited to collect blood samples for the serological examination were different from those visited to collect tissue samples. Assuming that each pig tissue sample came from a distinct animal, each sample was examined separately. The selection of tissue samples for both village chickens and pigs was based on the availability of organs in markets and farms, and the predilection sites of *Sarcocystis* reported by previous studies (Prakas & Butkauskas, 2012; Ng *et al*., 2015). Each sample was placed in a plastic bag and labelled. All samples were kept in a –20°C freezer.

Sample processing and DNA extraction

The samples were defrosted, and fat tissues were removed. The samples were then placed in a stomacher bag (Stomacher 400, Seward, UK) with 10ml of phosphate-buffered saline buffer (PBS pH 7.4, ThermoFisher Scientific, USA). The contents of the bag were homogenised with a stomacher (BagMixer Interscience, France) for 2 minutes at a high speed setting. Subsequently, 200µl of liquid and its contents were transferred into a new 2ml microcentrifuge tube for DNA extraction using the Geneaid Genomic Tissue DNA Kit (Geneaid Biotech Ltd., Taiwan) following the manufacturer's instructions.

PCR amplification, cloning and sequencing

Conventional PCR was carried out to amplify approximately 486 bp of the 18S rRNA gene and 800 bp of the first internal transcribed spacer (ITS1) fragment of *Sarcocystis*. PCR was carried out using primers 3L (CTAGTGATTGGAATGATGGG) and 3H (GGCAAATGCTTTCGCAGTAG) (Sun *et al.* 2021), and P-ITSF (ATTGAGTGTTCCGGTGAATTA) and P-ITSR (ITSR-GCCATTTGCGTTCAGAAATC) (Kutkienė *et al*., 2010) that were used to detect the genus *Sarcocystis* spp. in sheep and duck, respectively. The 25µl reaction mixtures contained 12µl of 2x TopTaq Master Mix Kit (Qiagen, Germany), 1µl of 0.2µM of each primer, 5µl of DNA template and 6µl of ddH_2O. The PCR conditions for the amplification of the 18S rRNA gene consisted of an initial denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 40 seconds, annealing at 57°C for 30 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 6 minutes. The ITS1 fragment was amplified with an initial denaturation of 95°C for 10 minutes, 35 cycles of denaturation at 94°C for 45 seconds, annealing at 58°C for 45 seconds, extension at 72°C for 2 minutes and a final extension at 72°C for 10 minutes. Each PCR cycle comprised a positive control (*Sarcocystis* DNA obtained from wild rats) and a negative control (RNase-free water). The PCR products were separated on a 1.5% agarose gel by electrophoresis, and Gel Doc XR Plus (Bio-Rad, USA) was used to visualise the results. A marker of 100bp DNA ladder (Qiagen, GmbH, Germany) was used.

Ten random positive amplicons of *Sarcocystis* 18S rRNA and ITS1 gene fragment samples each were selected, excised from the agarose gel, and purified using the QIAquick Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's protocol. The positive amplicons were cloned using the CloneJET PCR Cloning Kit (ThermoFisher, USA) in accordance with the manufacturer's instructions, and then plated on LB agar plates containing ampicillin. Using the aforementioned primers, direct colony PCR was used to screen ten colonies per plate for the presence of gene insertion. The 18S rRNA and ITS1 gene sequences were sent for commercial sequencing, and they were compared to known generic sequences in the GenBank database maintained by the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov).

Data collection

A closed-ended questionnaire was created with the aim of gathering data from the farms. The original questionnaire included a series of questions about possible farm-level risk factors for *Sarcocystis* infection in livestock farms that was taken from *T. gondii* published works (Herrero *et al.*, 2016; Stelzer *et al.*, 2019). To assess the understanding of the questions, detect potential problems, and determine how long it would take to complete the questions, a preliminary pilot study was conducted on a select few farms. The final questionnaire was designed to gather data on farm management, farm demography, and farm biosecurity measures. The comprehensive questionnaire was provided as supplemental information (Table S1).

Data analyses

The serological and molecular prevalence of *Sarcocystis* positivity were calculated by dividing the number of positive samples by the total number of samples. Using their respective 95% confidence intervals (95% CI), prevalence of *Sarcocystis* infection at the animal-level (number of individual animals sampled) and farm-level (number of farms visited) was calculated. If at least one animal on a farm has tested positive, the farm is considered positive. The Pearson Chi-square test (or Fisher's exact test) was used to cross tabulate the results of the serological analysis with the probable risk factor. Due to the possible clustering of data within a farm, multilevel logistics was taken into consideration for further study of the risk factors as suggested by Crowson (2020). However, the intra-cluster correlation (ICC) indicates a lack of reliability and that multilevel logistic is not appropriate for this data analysis. Therefore, risk factors that had a univariate significance level of $\alpha \leq 0.2$ (Deng *et al.*, 2016) were chosen and subjected to multivariable logistic regression analysis using the backward Wald method. The Haldane correction (observed frequency +0.5) was used for the odds ratio calculation when one of the cells had a zero value (Neuenschwander *et al.*, 2000). IBM SPSS Statistics (IBM, Armonk, NY: IBM Corp.) was used to conduct the analyses, and a significance level of α=0.05 was used.

After manually adjusting the DNA sequence electropherograms obtained from the clones using BioEdit v7.0.9 (Hall *et al*., 2011) to resolve ambiguity, phylogenetic analysis was performed. The derived sequences were then subjected to multiple alignments using ClustalX (Larkin *et al.*, 2007) to obtain consensus sequences. If a sequence was present in at least two clones, it was regarded as a unique haplotype. The nucleotide sequences were subjected to phylogenetic analysis using MEGA11 (Tamura *et al.*, 2021). All positions containing gaps and missing data were eliminated. Phylogenetic affinities were determined by constructing a Maximum Likelihood (ML) tree (Felsenstein, 1992) employing the Tamura-3-parameter with discrete Gamma distribution (+G) with 5 rate categories (for *Sarcocystis* 18S rRNA) and Hasegawa-Kishino-Yano with discrete Gamma distribution (+G) with 5 rate categories (for *Sarcocystis* ITS1). The strength of the phylogenetic trees analysis was tested using 100 bootstrap replicates (Felsenstein, 1992).

RESULTS

Descriptive statistics of farms

An overall of 250 and 433 sera samples were obtained from 25 village chicken farms and 19 pig farms, respectively. Selangor provided the most samples of chicken, while Penang and Melaka provided the most samples of pigs. A free-range farming method was utilised by most of the chicken farms examined in this study (88%) while an open-house farming system was used by most of the farms to raise pigs (89.47%).

Seroprevalence and risk factors of *Sarcocystis*

Sarcocystosis was discovered in over 35% of the farms inspected, however less than 10% of the animals on each farm had the disease (Table 1). The univariable analysis failed to detect any significant risk factors associated with the presence of *Sarcocystis* in the village chickens. On the other hand, the risk factors for infection in pigs were associated with 'uncovered feed storage' and 'wild animal access to feed and water' (Table 2). Multivariable logistic analysis found 'uncovered feed storage' significantly increased the probability of seropositivity at the level of the pig farm by 27.5 times (95% CI: 2, 378.84).

Molecular prevalence, haplotypes and phylogenetic tree of *Sarcocystis*

Sarcocystis DNA was detected in 16 out of 250 (6.4%; 95% CI: 4.6-11.6) village chicken samples but none in pork samples. No macrocysts were observed on the collected tissue samples. The 18S rRNA isolates showed a high degree of similarity (99%) with other *Sarcocystis* spp. deposited in GenBank. Meanwhile, the ITS1 region of *Sarcocystis* was similar to *Sarcocystis wenzeli* isolated from China (MT756996; 97-99% similarity) and *Sarcocystis* Chicken-2016-DF-BR

Table 1. Seroprevalence of *Sarcocystis* in village chickens and pigs in selected states in Peninsular Malaysia

Variable	Categories	No. animals tested	Positive (%)	95% CI	No. farms tested	Positive (%)	95% CI
Chickens	–	250	23 (9.20)	5.92–13.48	25	16 (64.00)	42.52-82.03
State	Perak	50	0	–	5	0	–
	Selangor	90	8 (8.89)	3.92–16.76	9	6 (66.67)	29.93–92.51
	Melaka	60	11 (18.33)	9.52–30.44	6	6 (100.00)	54.07–100.00
	Johor	50	4 (8.00)	2.22–19.23	5	4 (80.00)	28.36–99.49
Pigs	–	433	16 (3.70)	2.13–5.93	19	7 (36.84)	16.29–61.64
State	Penang	102	6 (5.88)	2.19–12.36	4	2 (50.00)	6.76–93.24
	Perak	100	1 (1.00)	0.02–5.45	4	1 (25.00)	0.60–80.59
	Selangor	28	0	–	3	0	–
	Melaka	102	7 (6.86)	2.80–13.63	4	2 (50.00)	6.76–93.24
	Johor	101	2 (1.98)	0.20–6.97	4	2 (50.00)	6.76–93.24

Table 2. Univariable analysis for risk factors in farm associated with the seroprevalence of *Sarcocystis* amongst pigs in Peninsular Malaysia

Risk factor	Categories	Frequency	Positive (%)	Chi-square (χ^2)	Odds ratio (95% CI)	p-value
States	Penang	4	3 (75.00)	2.00	9.00 (0.37–220.90)	0.49
	Perak	4	0.5 (12.50)	0.21	0.43 (0.01–17.83)	1.00
	Selangor	3	0.5 (16.67)	0.07	0.60 (0.01–26.47)	1.00
	Melaka	4	2 (50.00)	0.53	3.00 (0.15–59.88)	1.00
	Johor	4	1 (25.00)		1.00 [a]	
Farm system	Close	2	1 (50.00)	1.66	1.83 (0.20–34.85)	1.00
	Open	17	6 (35.30)		1.00[a]	
Type of feed	Produced on farm	6	3 (50.00)	0.65	2.25 (0.31–16.41)	0.38
	Commercial	13	4 (30.77)		1.00[a]	
Feed storage	Open	6	5 (83.33)	8.15	27.50 (2.00–378.8)	0.01
	Close	13	2 (15.38)		1.00[a]	
Feed location	On ground	3	1 (33.33)	0.02	0.83 (0.06–11.28)	1.00
	Off ground	16	6 (37.50)		1.00[a]	
Water source	Pipe	3	1 (33.33)	0.02	0.83 (0.06–11.28)	1.00
	Well	16	6 (37.50)		1.00[a]	
Farm often clean	Yes	17	6 (35.29)	0.17	0.55 (0.03–10.37)	1.00
	No	2	1 (50.00)		1.00[a]	
Presence of other livestock	Yes	5	1 (20.00)	0.83	0.33 (0.03–3.80)	0.60
	No	14	6 (42.86)		1.00[a]	
Presence of cat/dog	Yes	17	6 (35.29)	0.17	0.55 (0.03–10.37)	1.00
	No	2	1 (50.00)		1.00[a]	
Presence of rodents	Yes	14	5 (35.71)	0.03	0.83 (0.10–6.78)	1.00
	No	5	2 (40.00)		1.00[a]	
Contact with wild animals	Yes	8	4 (50.00)	1.03	2.67 (0.39–18.17)	0.38
	No	11	3 (27.27)		1.00[a]	
Other animals access feed and water	Yes	5	4 (80.00)	5.43	14.67 (1.16–185.24)	0.04
	No	14	3 (21.43)		1.00[a]	
Rodent control program	Yes	18	7 (38.89)	0.02	1.27 (0.04–43.18)	1.00
	No	1	0.5 (50.00)		1.00[a]	

[a] Reference category.

from Brazil (MN846302; 97-99% similarity). A total of 11 unique haplotypes, consisting of two haplotypes (SspMH01 and SspMH02) from the 18S rRNA gene and nine haplotypes (SspMH03-011) from the ITS1 region, were isolated from village chicken tissue samples. The obtained DNA sequences of the 18S rRNA and ITS1 genes were deposited in the GenBank database under accession numbers OP482179-OP482180 and OP490606-OP490614, respectively.

DISCUSSION

Wide variation in the prevalence of *Sarcocystis* infection have been documented in other animals in Malaysia including goats, water buffaloes, and cattle, ranging from 36% to 90% using various diagnostic techniques (Latif *et al*., 2013; Kutty *et al*., 2015; Zainalabidin *et al*., 2022). The current study revealed a relatively low animal-level prevalence of *Sarcocystis* infection in village chickens and pigs diagnosed serologically and molecularly. Comparing results from various studies is challenging because of the differences in study designs, diagnostic methods, and diverse methodologies employed. Antibodies to *Sarcocystis* sp. were detected in other avian species such as geese and psittacine birds (Cray *et al*., 2005; Konell *et al*., 2019). However, there are no studies reporting the detection of antibodies against *Sarcocystis* in chickens. The molecular prevalence of *Sarcocystis* in village chickens in the current study was higher than that reported in free-ranging chickens in Egypt (0.79%) through macroscopic and microscopic examination of the heart, liver, lung, and intestines (Galila *et al.*, 2021). However, a much higher prevalence was reported in free-range chickens from China (42.4%) following microscopic examinations of skeletal and heart muscles (Pan *et al.*, 2020).

The seroprevalence was similar but slightly higher than the molecular prevalence of *Sarcocystis* in the village chickens reported in this study. In experiments on sheep, researchers have demonstrated that the sarcocysts do not start to form in the muscle until one month and only begin to mature at four months following exposure to *Sarcocystis* infection (Toole, 1969; Munday & Obendorf, 1984). Moreover, in an experimental study on mice, antibodies against *Sarcocystis* can be detected using ELISA as early as 18 days post infection (Tenter, 1988). Therefore, our ELISA test may have only detected the presence of antibodies at the early stage of infection before the development of cysts in the chickens.

In pigs, the animal-level seroprevalence of *Sarcocystis* in this study was lower than a study of plasma samples among commercial sows in Germany via ELISA detection (29%) (Damriyasa *et al*., 2004). Farm-level seroprevalence in this study is similar to that in the aforementioned study in Germany (72%; Damriyasa *et al*., 2004). Other studies have detected *Sarcocystis* in pigs via PCR amplification (Făt *et al*., 2017; Espindola *et al.*, 2022), however, our study did not. In Malaysia, a higher prevalence of *Sarcocystis* (58%) has been previously reported in the heart, thigh, and oesophagus muscles of commercial pigs that were sampled from local abattoirs using pepsin digestion examination (Zainalabidin *et al.*, 2017). We were not able to detect the cysts in our pork samples, which is expected given the low level of seroprevalence reported in our study and routine post-mortem inspection of commercially slaughtered pigs for human consumption at local abattoirs. In addition, low parasite burden led to insufficient DNA yield, which would go undetected by PCR amplification.

In our previous report on the seroprevalence of *T. gondii* utilising the same sample (Leong *et al*., 2023), co-infections of *Sarcocystis* and *T. gondii* in village chickens and pigs detected using ELISA tests were 3.20% and 0.90%, respectively. According to Lind *et al*. (1997), these authors observed a moderate cross-reactivity response between the two aforementioned parasites in a lab experiment involving mice. Although we did not analyse the potential serological cross-reactivity of *Sarcocystis* spp. with *T. gondii,* cross-reactivity of antibodies may have occurred and the seroprevalence in the studied animals in this study may have been overestimated.

Uncovered storage of feed was identified as a significant driver of *Sarcocystis* infection in pig farms in the multivariable analysis. This practise allows complete access to feed for various vermin such as rodents, flies, and cockroaches. In addition, our univariable analysis showed that other wild animals that can easily access to water reservoirs and feed on the farm is statistically significant, which highlights the pertinent role of sporocyst contamination of feed and water in the transmission of sarcocystosis to farm animals. This is possible as open-house pig farms are open-sided and have low bio-security measures, therefore, vermin are free to enter the pigpen. Rodent species are known to be invasive and widespread, and are reported to play an important role as transmitters of other coccidian parasites such as *Cryptosporidium* spp. (Hancke & Suárez, 2022) and *T. gondii* (Junior *et al*., 2020). Therefore, it is not surprising that they can facilitate the perpetuation of *Sarcocystis* infection in pig farms by carrying sporocysts on their bodies and contaminating the feed, or harbouring the cysts in their tissue and gets eaten by the pigs. In addition, numerous studies have suggested the role of insects such as flies and cockroaches as mechanical vectors for spreading and maintaining the *Sarcocystis* infection in animals (Markus, 1980; Clubb & Frenkel, 1992). In studies conducted in the United States and England, cockroaches and flies were found to harbour *Sarcocystis* sporocysts on their legs, bodies, or mouthparts (Smith & Frenkel, 1978; Markus, 1980). According to Smith & Frenkel (1978), laboratory experiments have demonstrated that *Sarcocystis* oocysts remained infectious on cockroaches for a minimum of 20 days post exposure.

The study of *Sarcocystis* genetic variants is scarce. The overall number of haplotypes in this study is similar to a study of *Sarcocystis* isolated from Malaysian cattle based on the 18S rDNA gene (*n*=9) (Ng *et al.*, 2015). 18S rRNA and ITS1 genes are frequently used for genetic studies and identifying *Sarcocystis* species in various livestock animals. In our study, the phylogenetic analyses of the *Sarcocystis* 18S rRNA gene revealed a lack of discrimination strength to distinguish *Sarcocystis* species that belong to the genus (Figures 1), while ITS1 region phylogenies produced better genetic resolutions (Figures 2), corroborating its superiority as a genetic marker compared to 18S rRNA. Similar findings were also noted in other *Sarcocystis* spp. studies infecting avian species (Gjerde *et al.*, 2018; Prakas *et al.*, 2023). This is because the ITS1 gene has a more variable locus owing to the higher amount of indels such as insertions and/or deletions than the 18S rRNA gene, hence containing more important phylogenetic data to differentiate species within the genus (Acosta *et al*., 2021; Prakas *et al*., 2023). *Sarcocystis wenzeli* infection in this study was confirmed by ITS1 gene sequencing and phylogenetic trees; the analyses revealed six variants clustered with those from China and Brazil. Interestingly, the other three ITS1 isolates shared a stronger genetic affinity with *S. rileyi* from Norway, which is frequently found in ducks (Gjerde, 2014; Prakas *et al.*, 2023). This discovery may represent a chance of cross-species transmission of *Sarcocystis* from other avian species to the local village chickens. This is not surprising as other reports have suggested low host specificity of the *Sarcocystis* spp. infecting birds (Olias *et al.*, 2011; Kutkiene *et al.*, 2012). However, a more in-depth molecular analysis is required to validate the identity of the species.

There are a few limitations in this study that must be considered. Our pigs and chickens were sampled when they were ready for the market, so they might not accurately represent the species at various ages. Furthermore, the farm's sample size is limited, which could result in a lack of ability to detect important risk factors. The confidence interval for the risk factors described was consequently wider, and it should be interpreted with the acknowledgement of the sample size limitation.

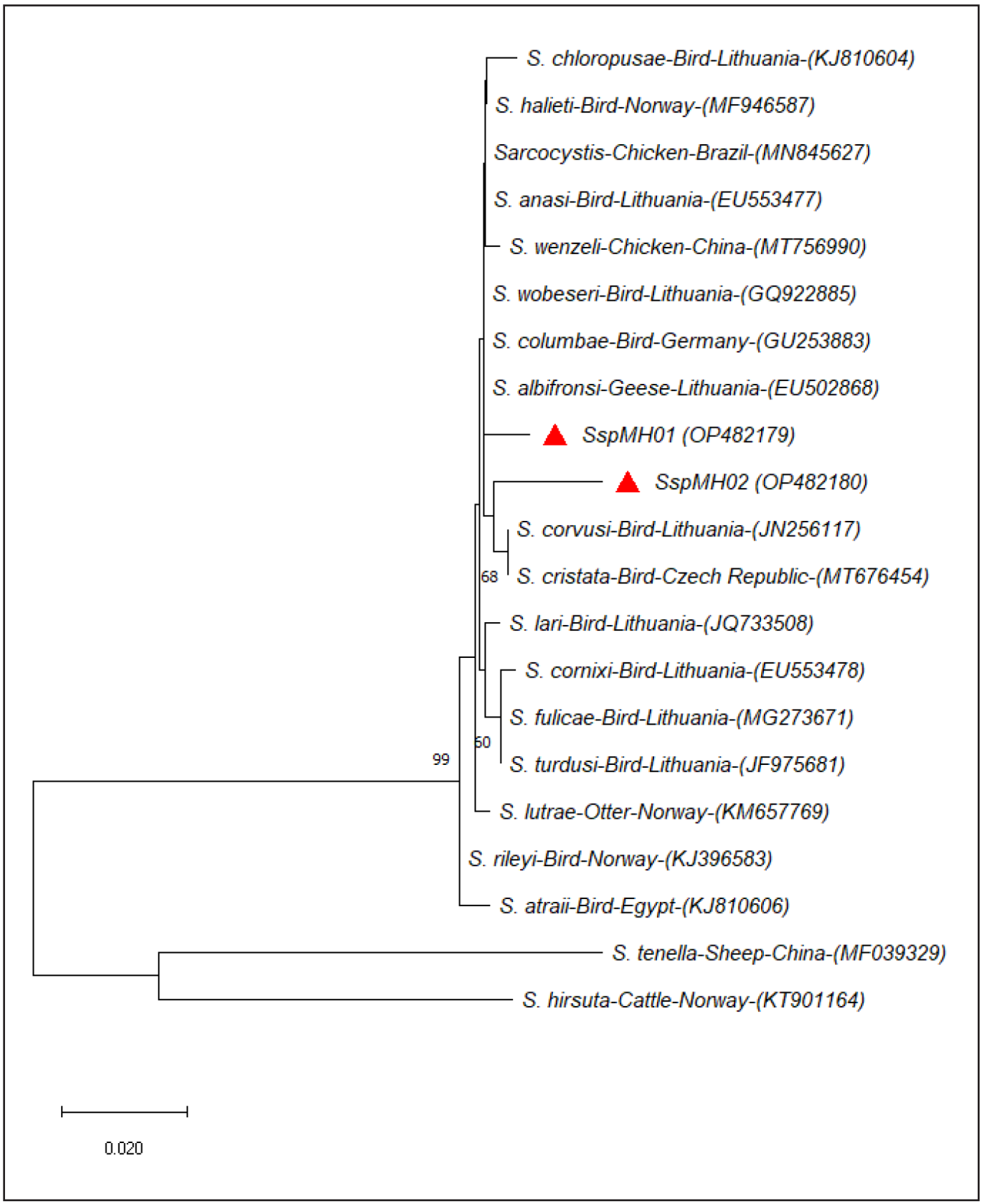

Figure 1. Maximum Likelihood phylogenetic tree of *Sarcocystis* based on 412 nucleotide residues of the 18S rRNA gene. The *Sarcocystis* haplotypes isolated in this study are marked with red triangles.

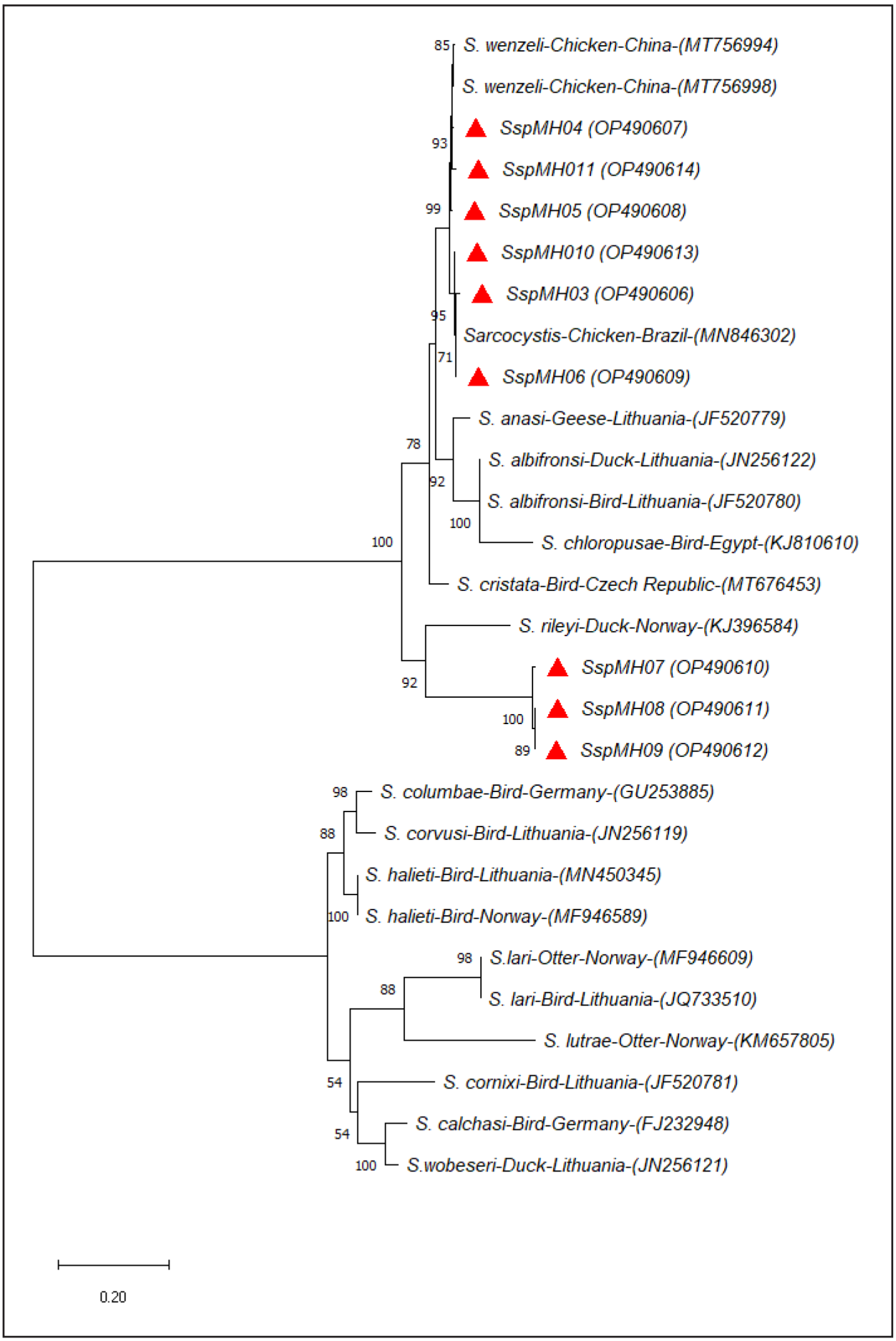

Figure 2. Maximum Likelihood phylogenetic tree of *Sarcocystis* based on 632 nucleotide residues of the ITS1 region gene. The *Sarcocystis* haplotypes isolated from this study are marked with red triangles.

CONCLUSION

We observed a low animal-level prevalence of *Sarcocystis* infection among village chickens and pigs compared to the reported prevalence from other studies. In village chickens, we report the detection of *Sarcocystis wenzeli* for the first time in Malaysia. Multivariable analysis of risk factor suggests that uncovered feed storage in pig farms contributed significantly to the seropositivity of sarcocystosis in pigs on the farm. Although the study is limited in sample size, the findings highlight a point of intervention that may positively further lower the risk of the parasite in pig farms, such as storing the feed in closed silos or containers impenetrable to other animals and insects.

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Conflict of interest

The author declares that they have no conflict of interest.

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