Research in Veterinary Science 103 (2015) 201–204

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/yrvsc

Short communication: Genetic variants of *Sarcocystis cruzi* in infected Malaysian cattle based on 18S rDNA

Yit Han Ng [a], Mun Yik Fong [a], Vellayan Subramaniam [b], Shahhaziq Shahari [a], Yee Ling Lau [a,*]

[a] Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
[b] Faculty of Pharmacy, Universiti Teknologi MARA, 43200 Puncak Alam, Selangor, Malaysia

ARTICLE INFO

Article history:
Received 21 March 2015
Received in revised form 28 September 2015
Accepted 25 October 2015

Keywords:
Sarcocystis cruzi
Malaysian cattle
18S rDNA
Intra-species variation

ABSTRACT

Sarcocystis species are pathogenic parasites that infect a wide range of animals, including cattle. A high prevalence of cattle sarcocystosis has been reported worldwide, but its status is unknown in Malaysia. This study focused on utilizing 18S rDNA to identify *Sarcocystis* species in Malaysian cattle and to determine their genetic variants. In this study, only *Sarcocystis cruzi* was detected in Malaysian cattle. The intra-species *S. cruzi* phylogenetic tree analysis and principal coordinate analysis (PCoA), respectively displayed two minor groups among the parasite isolates. This finding was supported by high Wright FST value (FST = 0.647). The definitive hosts (dogs) may play a fundamental role in the development of *S. cruzi* genetic variants. Additionally, the existence of microheterogeneity within the *S. cruzi* merozoites and/or distinct genetic variants arisen from independent merozoites in mature sarcocysts, possibly contributed to the existence of intra-species variations within the population.

Sarcocystis is a genus of protozoa belonging to the family Sarcocystidae. Like *Toxoplasma gondii* and *Neospora caninum*, *Sarcocystis* species are coccidian parasites with heterogeneous life cycles that require both intermediate and definitive hosts. Briefly, definitive hosts (predators) become infected when they consume infected intermediate hosts. In return, preys get infected when they consume the silages contaminated with the faeces containing oocysts of *Sarcocystis* spp. (Fayer et al., 2015). Livestock, including cattle, goats and sheep, are susceptible to sarcocystosis (Latif et al., 1999), and symptoms include fever, haemorrhages, low meat and milk yields, abortion, encephalomyelitis, and death in severe cases (Fayer et al., 2015).

Sarcocystis infections of muscle tissues (muscular sarcocystosis) are usually found in tongue, oesophagus, diaphragm, heart and skeletal muscles (Prakas and Butkauskas, 2012). The prevalence of cattle sarcocystosis is high in countries like Argentina (90%), (More et al., 2011) and Iran (98%) (Latif et al., 1999). However, the status of cattle sarcocystosis in Malaysia is unknown. There was only one report on the prevalence (40.8%) of sarcocystosis in Malaysian cattle and water buffaloes (Latif et al., 2013). There are multiple cattle breeds available in Malaysia including the indigenous Kedah-Kelantan (KK) breed, while the main imported cattle breeds, such as Brahman and Droughtmaster breeds, are from Australia. Although 85% of the total cattle population in Malaysia are KK breed (Johari and Jasmi, 2009), they are not preferred as meat producing resources due to small body masses and slower growth rate compared with imported cattle.

Currently, identification of *Sarcocystis* species relies on molecular phylogenetic analysis focusing on 18S rDNA (Yang et al., 2001; Rosenthal et al., 2008). 18S rDNA is ideal for phylogenetic studies because it is highly conserved, and analysis of its variable regions allows for effective speciation of organisms within a genus (Maidak et al., 1997). The aims of this study were to identify *Sarcocystis* species in Malaysian cattle via phylogenetic analysis of 18S rDNA sequences and to evaluate their genetic variants.

Muscle tissues of 11 local Malaysian cattle (*Bos indicus*) including the tongue, oesophagus, diaphragm, heart, and skeletal muscle, were collected from Abattoir Shah Alam, Selangor, Malaysia. Tissues were cut into about 2 cm^2 in area, and approximately 2 mm in thickness and stained with methylene blue for 10 min. For each sample, three pieces of tissues were cut at different spots in an attempt to increase the sensitivity for detecting sarcocysts. The stained samples were then compressed between two glass slides and were observed under a light microscope. Samples with sarcocysts were subjected to DNA extraction using a QIAGEN DNeasy Blood and Tissues Kit (QIAGEN, Valencia, CA, USA).

Two sets of primers notably 1L, 1H; and 3L, 2H, were used for the amplification of 18S rDNA via nested Polymerase Chain Reaction (PCR) (Yang et al., 2001). Amplicons were cloned into a pGEM-T Vector System (Promega, Madison, WI, USA) and transformed into TOP 10 competent cells. Positive clones were confirmed with insertions by colony PCR and were sequenced using M13 universal primers. All sequences were analysed and trimmed using Bioedit software (www.mbio.ncsu.edu/bioedit/bioedit.html) before undergoing BLAST analysis for species identification (MegaBlast). Three sequences corresponding to the same sample were further aligned to form a consensus sequence. These sequences were then subjected to multiple sequence alignment

* Corresponding author.
E-mail address: lauyeeling@um.edu.my (Y.L. Lau).

http://dx.doi.org/10.1016/j.rvsc.2015.10.009
0034-5288/

(ClustalW) with 20 other *Sarcocystis* species in GenBank prior to phylogenetic analysis (Supplementary Fig. S1).

Two phylogenetic trees were constructed based on the maximum-likelihood method as follows: 1) General *Sarcocystis* species tree; and 2) *Sarcocystis cruzi* intra-species tree. Both trees used a Tamura 3-parameter evolutionary model, where gaps and missing data were handled by complete deletion. The analytical method was chosen based on the best DNA substitution model MEGA-5 (Tamura et al., 2011). Besides, the nucleotide diversity and haplotypes of 18S rDNA and the Tajima neutrality test were calculated using DnaSP 5.10.00 (Rozas et al., 2003). The haplotypes were further analysed in principal coordinate analysis (PCoA) using GenAIEx v6.501 (Peakall and Smouse, 2012). Additionally, the Wright FST fixation index (Wright, 1951) in DnaSP 5.10.00 was used to calculate the genetic differentiation between the *Sarcocystis* spp. isolates.

All local cattle (n = 11) were positive for infection of *Sarcocystis* species under light microscopy, with at least one of the organs positive for sarcocysts. Among 55 tissues samples, 26 (47.3%) were detected positive for sarcocysts. Of these, only 76.9% (20/26) of them were amplifiable by PCR. This is likely either due to microscopic artefacts or the degradation of sample DNA prior to DNA extraction. Out of 20 sequences obtained, BLAST analysis indicated that 17 of them were *S. cruzi* whereas 3 were identified as *Theileria* species (Table 1). All *S. cruzi* isolates yielded an amplified fragment of the expected size, 948–962 bp. *S. cruzi* was detected in all organs and the infectivity rate of sarcocystosis corresponding to each organwas as follows: tongue = 29.4% (5/17); oesophagus = 23.5% (4/17); diaphragm = 11.8% (2/17); and heart and skeletal muscle were both recorded as 17.6% (3/17), respectively.

In diversity index analysis of *S. cruzi*, a total of 903 of 926 nucleotide sites (n = 26, comprising of 17 studied samples and 9 *S. cruzi* sequences in GenBank) were included, excluding 24 sites with gaps and missing data. Overall, 13 segregating sites were detected which resulted to 9 haplotypes. The haplotype diversity was recorded as $h = 0.7450 \pm 0.0650$ and the nucleotide diversity was $\pi = 0.0021 \pm 0.0005$. The Tajima neutrality test yielded a value of −1.7093, however the finding was not significant, $P > 0.05$ (Tajima, 1989). The details of the nucleotide polymorphisms were displayed in Supplementary Table 1.

The maximum likelihood-based tree separated all 47 sequences (17 current studied *S. cruzi* sequences; 9 *S. cruzi* reference sequences; 20 other *Sarcocystis* spp. sequences and *N. caninum* as outgroup) into four main clades corresponding to the definitive hosts, namely dogs/canids; cats; snakes; opossums and others (Supplementary Fig. S1). All studied *S. cruzi* sequences (n = 17) were distinguishable from other *Sarcocystis* species and clustered with the *S. cruzi* reference sequences (n = 9), under the dogs/canids clade. Additionally, the *S. cruzi* cluster formed a sister taxon with other three *Sarcocystis* species who also shared dogs or canids as their definitive hosts, notably *Sarcocystis capracanis*, *Sarcocystis arieticanis* and *Sarcocystis tenella*. Besides, the intra-species tree (Fig. 1A) displayed two minor clusters among the *S. cruzi* isolates, despite the fact that the bootstrap values were low. Likewise, the PCoA plot (Fig. 1B) analysis also successfully separated *S. cruzi* isolates into two minor clusters. The Wright's FST value between these two minor clusters was 0.647, indicating high genetic differentiation between these two groupings.

The 18S rDNA was used to differentiate and determine phylogenetic relationships between *Sarcocystis* species. In addition, we detected the tick-borne parasite *Theileria* species in the tongue and oesophagus. In contrast to a previous study on Malaysian cattle (Dissanaike and Kan, 1978), we did not detect *Sarcocystis hominis* or *Sarcocystis hirsuta*. One possible reason for the detection of *S. cruzi* rather than the detection of *S. hominis* or *S. hirsuta* in Malaysian cattle is the presence of dogs in farms. Dogs are the natural, definitive hosts of *S. cruzi*, and they excrete *S. cruzi* oocysts in their faeces (Dissanaike and Kan, 1978). Dog faeces containing the oocysts of *S. cruzi* may contaminate silages that cattle eat, thereby transmitting them to the cattle. In addition, flies may facilitate the transmission of the parasite from dog faeces to cattle feed (Markus, 1980). In contrast, *S. hominis* requires primates to complete its life cycle. Human (farmers) faecal contamination is thought to be rare as most farmers are well equipped with hygienic facilities such as toilets and washing stations in Malaysia. Likewise, *S. hirsuta* is transmitted by cats, but cats tend to bury their faeces, therefore the transmission of *S. hirsuta* is rarely detected in cattle (Latif et al., 2013).

Indeed, our finding shows that the phylogenetic and PCoA analyses were in concordance with high FST value [FST >0.25 is considered highly differentiated (Wright, 1978)], suggesting that the genetic variants of *S. cruzi* are likely to exist. However, our finding contradicts a previous report that highlighted the lack of genetic variability on *S. cruzi* 18S

Table 1
Summary of *Sarcocystis* species and other parasite found in infected Malaysian cattle (*Bos indicus*), Kedah-Kelantan breeds.

Organs/tissues	Isolate names	*Sarcocystis* species and other parasites	Intermediate host	Definitive host/vector	Amplified size (bp)	Accession numbers
Tongue	C20T	*S. cruzi*	Cattle	Dog	948	KJ917919
	C56T		(*Bos indicus*)	Family: Canidae	955	KJ917934
	C57T			(Dissanaike and Kan, 1978)	955	KJ917940
	C87T				955	KJ917949
	C88T				955	KJ917953
	C21T	*Theileria* sp.		Tick	914	KJ917962
	C58T			(*Rhipicephalus* sp.)	912	KJ917960
Oesophagus	C20E	*S. cruzi*	Cattle	Dog	952	KJ917909
	C56E		(*Bos indicus*)	Family: Canidae	955	KJ917925
	C60E			(Dissanaike and Kan, 1978)	955	KJ917943
	C90E				955	KJ917956
	C59E	*Theileria* sp.		Tick	911	KJ917965
				(*Rhipicephalus* sp.)		
Diaphragm	C20D	*S. cruzi*	Cattle	Dog	955	KJ917906
	C57D		(*Bos indicus*)	Family: Canidae	955	KJ917938
				(Dissanaike and Kan, 1978)		
Heart	C20H	*S. cruzi*	Cattle	Dog	962	KJ917912
	C56H		(*Bos indicus*)	Family: Canidae	955	KJ917929
	C87H			(Dissanaike and Kan, 1978)	955	KJ917948
Skeletal muscle	C20M	*S. cruzi*	Cattle	Dog	955	KJ917917
	C21M		(*Bos indicus*)	Family: Canidae	955	KJ917924
	C56M			(Dissanaike and Kan, 1978)	955	KJ917931