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Molecular detection of *Trypanosoma evansi* in dogs from India and Southeast Asia

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ABSTRACT

Trypanosoma evansi, the causative agent of surra, is a hemoflagellate protozoan mechanically transmitted by hematophagous flies, mainly in tropical and subtropical regions. This protozoan affects several mammalian hosts, including dogs, which are highly susceptible to the infection. To investigate the occurrence of *T. evansi* in dogs, a total of 672 DNA samples from India (n = 228), Indonesia (n = 57), Malaysia (n = 45), the Philippines (n = 103), Thailand (n = 120), and Vietnam (n = 119) were screened by using species-specific conventional PCR. Of the tested dogs, 10 (1.5%) scored positive to *T. evansi*. In particular, positive samples were detected in canine blood samples collected from India (n = 4; 1.8%), Indonesia (n = 4; 7%), and Malaysia (n = 2; 4.4%). All tested samples from the Philippines, Thailand and Vietnam were negative. Nucleotide sequence analysis revealed a high variation (i.e. from 0.4% to 6.2%) among the RoTat 1.2 variant surface glycoprotein (*vsg*) gene. Although the number of sequences included in this analysis is relatively small, this nucleotide variation may indicate the divergence of *T. evansi* RoTat 1.2 *vsg* gene among different strains. The high incidence of *T. evansi* previously reported in cattle and buffaloes in India and Southeast Asia suggests that these animals are the main source of infection to dogs.

1. Introduction

Trypanosomes are hemoflagellate protozoa transmitted by blood-feeding arthropods causing infections in a wide range of mammalian hosts including humans, mainly in tropical and subtropical regions (Radwanska et al., 2018). For instance, *Trypanosoma cruzi* is responsible for Chagas disease in the Americas while *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* cause human trypanosomoses in sub-Saharan African countries (Browne et al., 2017; Jackson et al., 2010). Among the pathogenic trypanosomes, *Trypanosoma evansi*, the causative agent of surra, has the widest host range and geographical distribution (Radwanska et al., 2018). Since its first description in blood of horses and dromedaries in India in 1880 Hoare (1972), *T. evansi* has been found affecting many domestic animals (e.g. buffaloes, camels, cattle, and dogs) and wildlife (e.g. bats, deer, and rodents) in South and Central America, North Africa, the Middle East, Indian subcontinent and Southeast Asia (SEA) (Aregawi et al., 2019; Nguyen et al., 2021). In India, the disease in cattle has an estimated economic impact of US $200.4 million per year, due to loss in milk production (Kumar et al., 2017). A very limited number of human cases by *T. evansi* infection have been reported in India and Vietnam with patients presenting intermittent fever and headache (Joshi et al., 2005; Van Vinh Chau et al., 2016). The broad host range and wide geographical distribution of this protozoan is due to its mechanical transmission through the bites of various hematophagous flies such as those belonging to the genera *Stomoxys* and *Tabanus* (Radwanska et al., 2018). Alternative transmission of *T. evansi* to mammalian hosts includes blood transfusion, feeding on fresh ~~infected~~ meat or carcasses, or in South America, through the bite of the common vampire bat *Desmodus rotundus* (Hoare, 1965; Sinha et al., 1971).

Trypanosomosis caused by *T. evansi* is considered endemic in India and Southeast Asian countries, such as Indonesia, Malaysia, the Philippines, Thailand and Vietnam, with frequency of infection reaching up to 70% in livestock (Davison et al., 2000; Kocher et al., 2015; Nguyen

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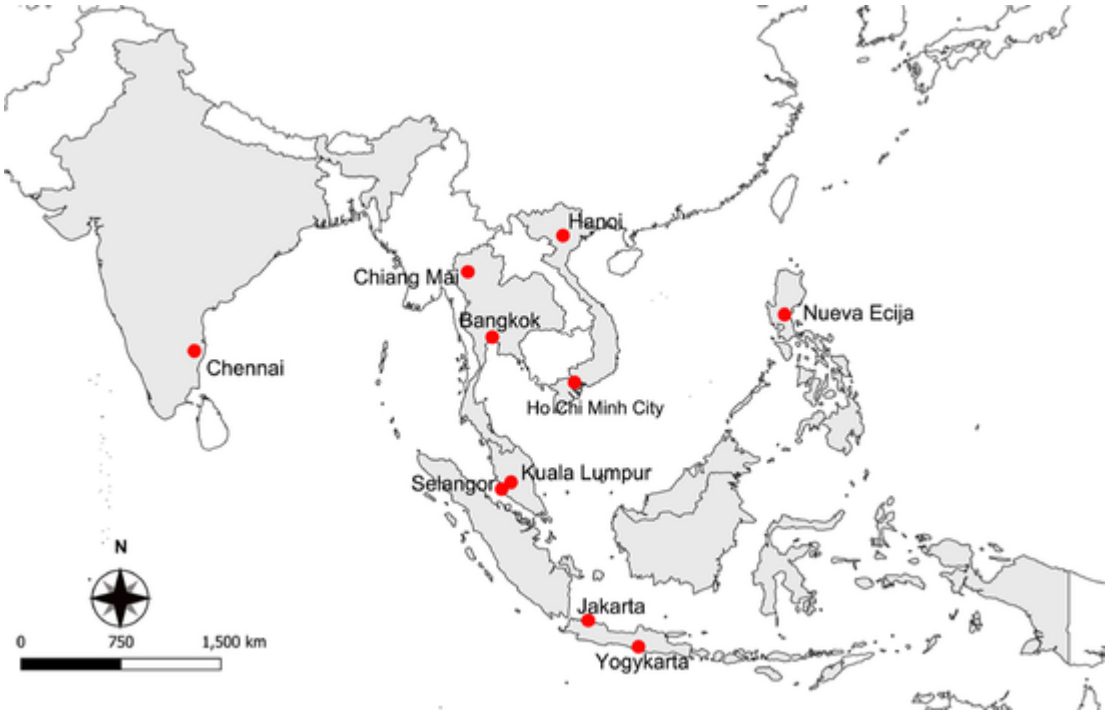

Fig. 1. Geographical distribution of dog samples in this study.

Table 1
Number and percentage of dogs positive to *Trypanosoma evansi* according to their sex and age.

Country	Sex		Age group			
	Male	Female	≤ 1	1 year < 5 years	≥ 5	NA*
India	3/112 (2.7%)	1/116 (0.9%)	0/12	1/125 (0.8%)	3/91 (3.3%)	0
Indonesia	3/29 (10.3%)	1/28 (3.6%)	1/15 (6.7%)	2/26 (7.7%)	1/16 (6.3%)	0
Malaysia	1/20 (5%)	1/25 (4%)	0/9	0/10	2/26 (7.7%)	0
Philippines	0/46	0/57	0/40	0/35	0/27	0/1
Thailand	0/70	0/50	0/15	0/37	0/66	0/2
Vietnam	0/65	0/54	0/46	0/46	0/24	0/3
Total	7/342 (2%)	3/330 (0.9%)	1/137 (0.7%)	4/279 (1.4%)	5/250 (2%)	0/6

*Not Available

et al., 2013; Ola-Fadunsin et al., 2020; Sharma et al., 2013). Among the domestic animals, dogs are highly susceptible to *T. evansi* (Desquesnes et al., 2013) and the infection may lead to significant clinical signs such as anemia, edema, corneal opacity, intermittent fever, lymphadenopathy (Bono Battistoni et al., 2016; Echeverria et al., 2019; Rjeibi et al., 2015) and death in acute forms (Bui et al., 2021; Greif et al., 2018; Jaimes-Dueñez et al., 2017). Clinical cases of canine trypanosomosis by *T. evansi* have been reported in different countries, including Argentina (Bono Battistoni et al., 2016), Brazil (Eloy and Lucheis, 2009), Colombia (Jaimes-Dueñez et al., 2017), Uruguay (Greif et al., 2018), Tunisia (Rjeibi et al., 2015), Afghanistan (Aref et al., 2013), Iran (Hosseininejad et al., 2007), India (Panigrahi et al., 2015), Sri Lanka (Dangolla et al., 2020), Thailand (Barameechaithanun et al., 2009), and Vietnam (Bui et al., 2021). Imported cases of canine trypanosomosis by *T. evansi* have been reported ~~also~~ in Europe (Defontis et al., 2012; Hellebrekers and Slappendel, 1981).

In endemic areas, data about the infection prevalence in dogs is underestimated, mainly because it relies on the detection of the protozoan in stained blood smear and microhematocrit test, which has a quite a low sensitivity (Prasad et al., 2015; Ravindran et al., 2008). To assess the prevalence of infection in dogs in enzootic areas, diagnostic method such as PCR should be applied for the detection of *T. evansi*, especially in the early stage of disease or in the case of subclinical infection (Azhahianambi et al., 2018; Claes et al., 2004; Ravindran et al., 2007). Hence, the aim of this study was to molecularly investigate the occurrence of *T. evansi* in dogs from India and Southeast Asia.

2. Materials and methods

2.1. Animal enrollment

A total of 672 blood samples of dogs originated from tropical regions of India and SEA (Fig. 1) collected under the frame of previous studies (Colella et al., 2020; Manoj et al., 2020) was screened for the presence of *T. evansi* DNA. In particular, samples were collected from 228 stray dogs from Tamil Nadu state (southern India) in 2018, and from 444 privately-owned dogs from Indonesia (n = 57), Malaysia (n = 45), the Philippines (n = 103), Thailand (n = 120) and Vietnam (n = 119) in 2017–2018. Data regarding age, sex, breed, and infestation by ectoparasites were also gathered on dogs apparently healthy at the time of sampling. Genomic DNA from whole blood samples (from India) and dried blood spots (from SEA) was isolated using GenUP Blood DNA Kit (Biotechrabbit, Germany) and QIAamp 96 DNA QIAcube HT Kit (Qiagen, Germany), respectively, according to the manufacturers' instructions.

2.2. Molecular assays

Trypanosoma evansi DNA was assessed by using conventional singleplex PCR, with species-specific primers (TevF: 5′-TGCAGACGACCTGACGCTACT-3′ and TevR: 5′-CTCCTAGAAGCTTCGGTGTCCT-3′) to amplify a portion of 227 bp of the variant surface glycoprotein (*vsg*) gene of *T. evansi* Rode Trypanozoon antigenic type (RoTat) 1.2 (Azhahianambi et al., 2018). The following conditions were used: 10 min pre-denaturation at 95 °C, followed by 35 amplification cycles (95 °C for 30 s, 64 °C for 30 s and

Table 2
Data of dogs infected by *Trypanosma evansi*, and the GenBank accession number of the obtained partial RoTat 1.2 *vsg* gene nucleotide sequences with percentage of nucleotide identity.

Sample ID	Origin	Age (year)	Sex	Breed	Ectoparasites	Lifestyle	Accession number	Nucleotide identity
CI-D54	Chennai, India	11	Male	Rajapalayam	*Rhipicephalus sanguineus*	Stray	MW489877	97.8% – JQ653273
CI-D126	Chennai, India	8	Male	Crossbreed	*Rhipicephalus sanguineus*	Stray	MW489878	97.35% – MG600142
CI-D165	Chennai, India	4	Female	Crossbreed	*Rhipicephalus sanguineus*	Stray	MW489879	97.78% – MF981024
CI-D181	Chennai, India	5	Male	Crossbreed	*Rhipicephalus sanguineus*	Stray	MW489880	98.22% – MF981024
JI-D16	Jakarta, Indonesia	1	Male	Beagle	*Rhipicephalus sanguineus*	Privately-owned	MW489881	99.56% – MF981024
JI-D35	Jakarta, Indonesia	3	Male	Crossbreed	-	Privately-owned	MW489882	98.23% – MG600142
YI-D52	Yogyakarta, Indonesia	3	Female	Crossbreed	*Ctenocephalides* spp. *Rhipicephalus sanguineus*	Privately-owned	MW489883	96.92% – MK757490
YI-D60	Yogyakarta, Indonesia	5	Male	Crossbreed	*Rhipicephalus sanguineus*	Privately-owned	MW489884	100% – MG600142
SM-D01	Kuala Lumpur, Malaysia	10	Male	Crossbreed	-	Privately-owned	MW489885	100% – MF981024
SM-D31	Kuala Lumpur, Malaysia	11	Female	Crossbreed	-	Privately-owned	MW489886	96.02% – MG600142

Table 3
The divergence (%) of the obtained *Trypanosma evansi* RoTat 1.2 *vsg* nucleotide sequences.

Sample ID		1	2	3	4	5	6	7	8	9	10
CI-D54	**1**										
CI-D126	**2**	3.96									
CI-D165	**3**	3.96	5.29								
CI-D181	**4**	3.52	4.85	0.44							
JI-D16	**5**	3.08	3.52	2.20	1.76						
JI-D35	**6**	3.96	2.64	4.41	3.96	2.64					
YI-D52	**7**	3.52	5.73	3.96	4.41	3.96	4.41				
YI-D60	**8**	2.20	2.64	2.64	2.20	0.88	1.76	3.08			
SM-D01	**9**	2.64	3.08	2.20	1.76	0.44	2.20	3.52	0.44		
SM-D31	**10**	4.41	5.73	5.73	5.29	4.85	5.73	6.17	3.96	4.41	

72 °C for 30 s) and a final extension at 72 °C for 5 min. DNA of *T. evansi* AnTat 3/3 strain was used as positive control for each PCR reaction. Amplified PCR products were examined in 2% agarose gels stained with GelRed (VWR International PBI, Milan, Italy) and visualized on a GelLogic 100 gel documentation system (Kodak, New York, USA). The PCR amplicons were then purified and sequenced in both directions using the same primers as for PCR, employing the Big Dye Terminator v.3.1 chemistry in a 3130 Genetic Analyzer (Applied Biosystems, California, USA). The obtained nucleotide sequences were aligned and edited using the BioEdit 7.0 software and compared with those available in the GenBank database using Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Nucleotide identity comparison using pairwise method with the p-distance model was conducted. The transition/transversion ratio (*R*) among sequences was calculated using the Kimura 2-parameter substitution model. The nucleotide sequences were conceptually translated into amino acid sequences using the protozoan mitochondrial code to select the proper open reading frame. Phylogenetic relationships were inferred using the Maximum Likelihood (ML) method based on Kimura 2-parameter model with discrete Gamma distribution to model evolutionary rate differences among sites selected by best-fitting model. Evolutionary analysis was conducted with 1000 bootstrap replications. All nucleotide sequences and phylogenetic analysis were performed by using MEGA X software (Kumar et al., 2018). Homologous sequence from *Trypanosoma brucei* (M28525) was used as an outgroup. Additionally, Bayesian analysis were performed using the program MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001) with 2,000,000 generations. The first 25% of these trees represented the "Burn in" and the rest of the trees was used to calculate Bayesian analysis.

2.3. Data analysis

Exact binomial 95% confidence intervals (CI) were established for proportions. Data analysis ~~were~~ performed using IBM SPSS Statistics 16.0 software. The possible associations between proportion of animal infected and risk factors such as sex, age (≤ 1 year, > 1 to < 5 years, and ≥ 5 years), and origin were analyzed using Fisher's exact test with p value < 0.05 considered as statistically significant.

3. Results

Overall, *T. evansi* was detected in 1.5% (95% CI: 0.8–2.7%) (10/672) of canine blood samples and more specifically in dogs from India ($n = 4$; 1.8%; 95% CI: 0.6–4.5), Indonesia ($n = 4$; 7%; 95% CI: 2.4–17.3) and Malaysia ($n = 2$; 4.4%; 95% CI: 0.8–15.2) (Table 1). None of the tested samples from the Philippines (0%; 95% CI: 0–3.6), Thailand (0%; 95% CI: 0–3.1) and Vietnam (0%; 95% CI: 0–3.1) scored positive. No statistically significant correlations were found between *T. evansi* positive dogs and their sex ($p = 0.341$), age ($p = 0.741$), and origin ($p = 0.065$). The presence of ectoparasites was reported in eight out of 10 positive dogs, with the brown dog tick *Rhipicephalus sanguineus* sensu lato found on seven and fleas of the genus *Ctenocephalides* on one dog (Table 2).

A portion of the RoTat 1.2 *vsg* gene of *T. evansi* was amplified from all PCRs positive samples. BLAST analysis of the obtained sequences revealed 96–100% nucleotide identity with those available in the GenBank database (accession numbers: JQ653273, MG600142, MF981024, MK757490). Pairwise comparison among obtained sequences revealed nucleotide variation ranging from 0.4% to 6.2% (Table 3). In particular, the mean intraspecific differences recorded among sequences generated from samples in India, Indonesia, and Malaysia were 3.7% (range: 0.4–5.3%), 2.8% (range: 0.9–4.4%), and 4.4%, respectively. Nearly half of the nucleotide variability ($n = 13$; 48.1%) was at the third codon position, whereas the remainders were at the first ($n = 8$; 29.6%) and second ($n = 6$; 22.2%) positions. The nucleotide variation was also represented by transitions rather than transversions, with R ratio of 2.3. The nucleotide substitutions encoded amino acid sequences without stop codon. The sequences of *T. evansi* RoTat 1.2 *vsg* gene herein generated were clustered in the same clade with significative bootstrap value as shown in the ML tree (Fig. 2). ~~Indeed, all~~ *T. evansi* sequences in this study clustered with other *T. evansi* from different hosts from India and the Middle East. In addition, the Bayesian analysis also confirmed the same cladding with a high bootstrap value. All sequences generated in this study were deposited in the GenBank database under the accession numbers MW489877–MW489886.

4. Discussion

This study reports the occurrence of *T. evansi* in dogs from India, Indonesia and Malaysia. Although the prevalence of *T. evansi* herein detected in dogs from India (i.e. 1.8%) is similar to that recorded in a previous study (2.7%; Vismaya et al., 2020), higher infection rates (i.e. 21%) were detected also in dogs suspected to hemoprotozoan infection in this country (Azhahianambi et al., 2018). In Malaysia, *T. evansi* was

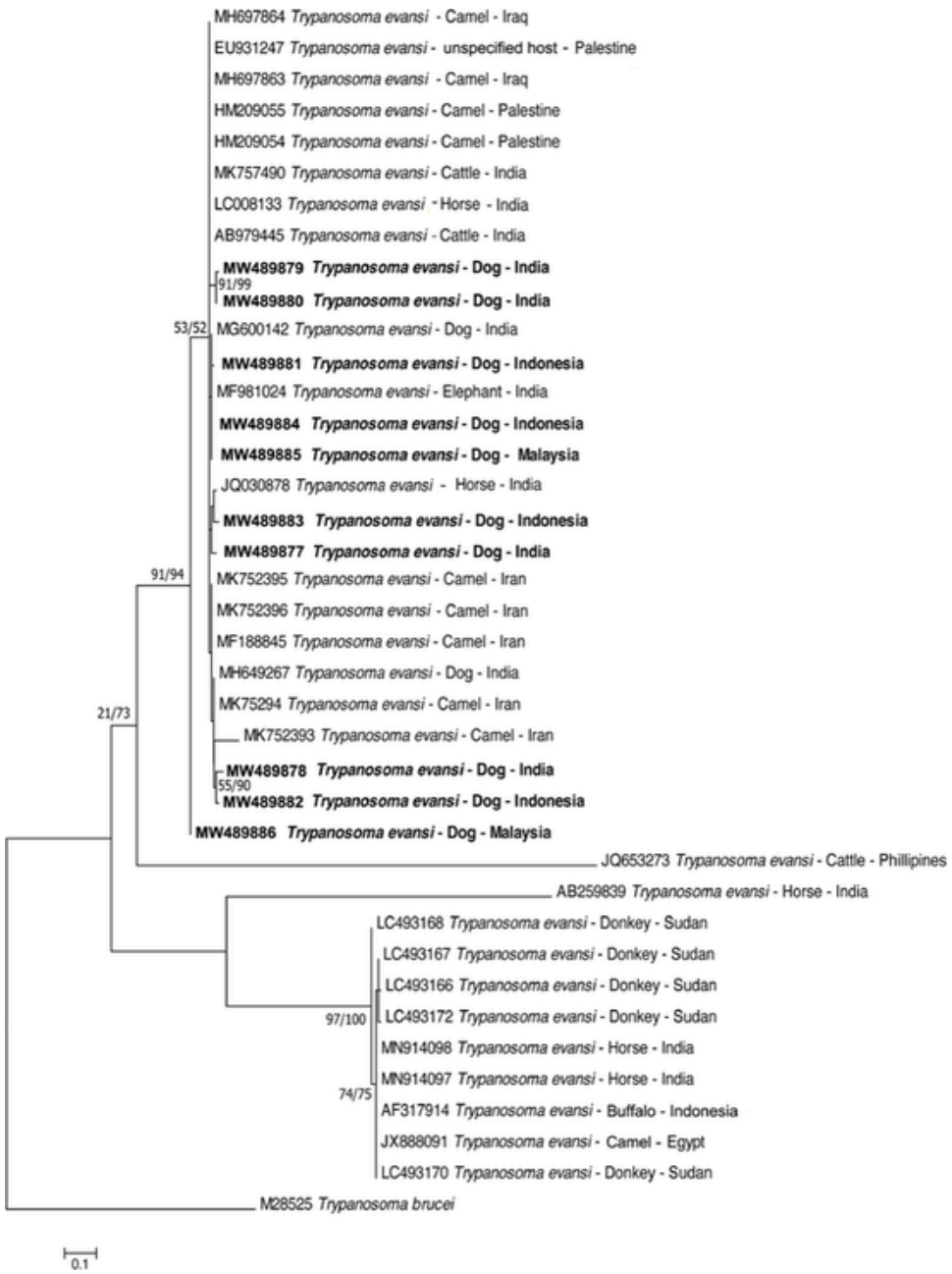

Fig. 2. Maximum Likelihood tree inferred from RoTat 1.2 *vsg* nucleotide sequences of *Trypanosoma evansi*, using *Trypanosoma brucei* as outgroup, with indication of the nodal support from Bayesian inference analysis. Numbers at nodes are the support values ordered as ML/BI. The sequences obtained in this study are indicated in bold.

only detected once in dogs by using Giemsa stained blood smear (Rajamanickam et al., 1985); therefore, this is the first molecular report of this protozoan infection in dogs from Malaysia as well as from the neighboring country Indonesia. Although cases of canine *T. evansi* infections were previously reported in Thailand and Vietnam (Barameechaithanun et al., 2009; Bui et al., 2021), the absence of this parasite among dogs in these countries could be due to the low prevalence of the infection. In addition, the target gene herein used might represent a limitation, since the gold standard PCR (i.e., TBR1/2 PCR that amplifies repetitive sequences of the mini-chromosome satellite) for *Trypanozoon* DNA detects as few as 1–5 *Trypanosomes*/ml of blood, whereas the RoTat 1.2 *vsg* PCR no less than 50 *Trypanosomes*/ml of blood (Tehseen et al., 2015).

The source of *T. evansi* in dogs from India and SEA is more likely from infected livestock of which high prevalence have been recorded. For instance, cattle from Malaysia and India have been reported with prevalence up to 20% and 36.5%, respectively (Ola-Fadunsin et al., 2020; Sharma et al., 2013). Similarly, high prevalence of *T. evansi* infection have been recorded in buffaloes from Thailand (12.2%; Kocher et al., 2015), Vietnam (22.4%; Nguyen et al., 2013), Indonesia (70%; Davison et al., 2000), and the Philippines (90%; Dargantes et al., 2009). Therefore, livestock act as reservoir and play an important role in the maintenance of this parasite in endemic areas (Desquesnes et al., 2013; Herrera et al., 2004).

In domestic dogs, *T. evansi* may be transmitted by hematophagous flies as in livestock, but also through oral mucosa penetration of the parasite during the ingestion of contaminated blood or consumption of infected rodents (Panigrahi et al., 2015; Raina et al., 1985). Moreover, alternative sources of *T. evansi* infection to dogs by consuming contam-

inated raw beef should also be considered (Morelli et al., 2019; van Bree et al., 2018).

Although the brown dog tick *R. sanguineus* s.l. was found on 7 out of 10 *T. evansi*-positive animals, the role of this tick species as passive vector of this protozoan was not demonstrated (Vergne et al., 2011), probably because the mechanically transmission of *T. evansi* requires a shorter time frame than that ~~in between two hosts feeding of ticks~~ Otranto (2018).

The high nucleotide variation herein registered within our sequences may reflect the divergence of *T. evansi* RoTat 1.2 *vsg* gene among different strains, as it was also observed among those obtained from different hosts and geographical areas (Gaur et al., 2020). This could be the result of an immune escape strategy of trypamosomes to cope with host-immunity by switching VSG coat antigens. Indeed, RoTat 1.2 is a predominant VSG expressed by most strains of *T. evansi* (except those isolated from Kenya) during the early, middle and late phases of the infection (Ngaira et al., 2004; Verloo et al., 200 herefore, it has been employed as the sensitive and specific target for the serological as well as molecular detection of *T. evansi* infection (Claes et al., 2004; Verloo et al., 200 he effect of host species from different geographical areas to the variation of the *T. evansi* RoTat 1.2 *vsg* gene deserves further investigations. In addition, along with the analysis of the ITS-2 gene (Areekit et al., 2008; Khuchareontaworn et al., 200 he RoTat 1.2 *vsg* gene could be useful in elucidating the genetic variability within *T. evansi* population.

5. Conclusion

This study provides more data on the circulation of *T. evansi* in dogs in endemic regions. The high incidence of *T. evansi* in cattle and buffaloes in India and Southeast Asia suggests that these animals are the main source of *T. evansi* infecting dogs in these areas.

Authors' contributions

Conceptualization: Domenico Otranto, Vito Colella, Viet-Linh Nguyen; Supervision: Roberta Iatta, Domenico Otranto; Methodology: Viet-Linh Nguyen, Roberta Iatta; Investigation: Ranju Ravindran Santhakumari Manoj, Viet-Linh Nguyen; Data curation: Viet-Linh Nguyen, Roberta Iatta, Jairo Alfonso Mendoza-Roldan; Writing - original draft: Viet-Linh Nguyen, Roberta Iatta; Writing - review & editing: Vito Colella, Marcos Antônio Bezerra-Santos, Ranju Ravindran Santhakumari Manoj, Domenico Otranto. All authors read and approved the final manuscript.

Declaration of Competing 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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