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Molecular detection of *Trypanosoma evansi* in cattle from Quirino Province, Philippines

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ABSTRACT

Trypanosoma evansi is a protozoan parasite which ubiquitously infects livestock in the Philippines. The study depicts an initial report of livestock trypanosomes in cattle from the province of Quirino, Philippines using the polymerase chain reaction (PCR) method. Out of 246 field blood samples from apparently healthy cattle collected from five municipalities of Quirino Province, Philippines, 3/246 (1.22%) were established positive for *T. evansi* using PCR. The positive animals identified were from the municipality of Saguday (3/42; 7.14% prevalence). These were composed of one female and two males species and belonged to variable age groups (1.1-2 yrs, 2.1-3 yrs and >3 yrs). In addition, the data on the physical examination of the sampled animals using the nine-point scale body condition scoring system showed that the top three body condition scores (BCS) were 3[Very thin, (107/246; 43.50%)], 4[Thin, (75/246; 30.50%)] and 2[Emaciated, (46/246; 18.70%)]. Furthermore, the data showed that majority of the animals in Diffun and Saguday were thin (BCS 4) while most of the animals in Cabarroguis and Maddela and Aglipay were classified under BCS 3(Very thin) and BCS 2(Emaciated) respectively. Consequently, all the *Trypanosoma evansi* positive animals appeared thin (BCS= 4) and were from the municipality of Saguday. No parasites were observed after blood parasite examination (BPE). The study provides information on the first report of *T. evansi* cases in cattle from Quirino Province located in Region 2 of the Philippines. Consequently the data also presents the first molecular detection of *T. evansi* infection in cattle in the province of Quirino, Philippines.

Key words: *Trypanosoma evansi*, surra, blood parasite, polymerase chain reaction, Quirino Province, Philippines

Introduction

One of the most common diseases of livestock that is of critical importance is caused by a protozoan parasite *Trypanosoma evansi*. The disease trypanosomosis is known locally as surra, "higpit" or "bayawak".

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Surra is an economically important vector-borne livestock disease since cattle and carabaos are raised for both meat and milk products in the country. Infection leads to poor reproductive performance, low meat and milk yield production as well as high mortality (MANUEL, 1998). The disease is allegedly present in various islands (MANUEL, 1998) of the Philippines causing sporadic cases that are widespread in the whole archipelago (LUCKINS, 1998). Tabanid flies or horseflies (*i.e. Tabanus striatus* and *T. reducens*), locally known as *palangat*, are known as the primary transmitters of the disease in the Philippines. In addition, the disease can also be transmitted mechanically by several blood-sucking flies like stable fly (*Stomoxys calcitrans*), buffalo fly (*Haematobia* spp.), deer fly (*Chrysops* spp.) and mosquitoes (MANUEL, 1998; SOULSBY, 1982). Over the past decade, the number and severity of surra outbreaks have increased dramatically, with cattle ranking third in terms of animal morbidity and mortality (REID, 2002; ANONYMOUS, 2002).

Several methods have been developed for the detection of *T. evansi* infection but among these, PCR is considered to be the most sensitive and specific and it has a wider range of application (REID, 2002; SUKHUMSIRICHART et al., 2000; MUGITTU et al., 2001; MORLAIS et al., 1998). In this regard, the present study explored the preliminary use of PCR in conjunction with parasitological examination method to detect surra in cattle from Quirino Province.

Out of the total of 246 field blood samples from apparently healthy cattle collected from five municipalities of Quirino province, 3/246 (1.22%) indicated positive for *T. evansi* after PCR assay. On the other hand, no parasites were observed after BPE.

The results of the study exhibited the applicability of polymerase chain reaction assay for epidemiological surveillance of trypanosomosis in the country. Furthermore, this study reports the first case of *Trypanosoma evansi* infection in cattle from Quirino province and also the first molecular evidence of *Trypanosoma evansi* infection in cattle in the province of Quirino, Philippines.

Materials and methods

Animals and sampling site. A total of 246 cattle (*Bos taurus*) were randomly sampled from five out of six municipalities of Quirino Province. The chosen municipalities included Diffun, Cabarroguis, Saguday, Aglipay and Maddela (Fig. 1). The sample size was determined using a formula previously described (THRUSFIELD, 1986). Signalment and other data regarding the history of the animal were obtained using a questionnaire. In addition, physical examination of each cattle was conducted following the nine-point scale body condition scoring (BCS) system as previously described (WAGNER et al., 1988) (Table 1).

Blood parasite examination. Blood smears were Giemsa® (Medic Diagnostic Reagents, Philippines) stained and examined following the method of PRITCHARD and KRUSE (1982) using a microscope (Olympus®, USA) at high power (40x) and oil immersion (100x) objectives.

DNA extraction. The genomic DNA extraction was performed based on previous reports (BATICADOS et al., 2005; BATICADOS et al., 2010a; 2010b). Briefly, one part of blood from each sample was mixed with nine parts DNA extraction buffer [0.2 M NaCl, 10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 1% SDS and 1/100 volume of proteinase K (Sigma-Aldrich, Inc., St. Louis, USA)] in individual 1.5 mL microcentrifuge tubes (Molecular BioProducts, Inc., San Diego, CA). The mixture was incubated at 55 ºC for 10-12 hours. After incubation, the resulting viscous solution was extracted once with phenol-chloroform-isoamyl alcohol (PCI) (Sigma-Aldrich, Inc., St. Louis, USA) and once with chloroform, followed by centrifugation at 15000 rpm for five minutes after each extraction. The extracted DNA was precipitated with 3M sodium acetate and 99.5% ethanol, followed by 70% ethanol wash. The recovered DNA pellets were suspended in 20µL triple distilled water and stored at -40 ºC.

Polymerase chain reaction (PCR). The DNA extracts were subjected to PCR assay. The sequence of the species-specific primers are detailed in Fig. 2a.

The total DNA concentration of the samples was measured using a spectrophotometer (NanoPhotometer™, Implen GmbH, Munich, Germany). The diluted DNA template (1µg) was transferred into a PCR tube and the PCR mixture composed of 10x PCR buffer, 2mM dNTP mixture, *Trypanosoma evansi* primer pair [10 pmoles each (Invitrogen, Singapore)], triple distilled water and Taq polymerase (iNTRON Biotechnology, Inc., Korea) was added. PCR amplification was carried out using a thermal cycler (C1000™ Thermal Cycler, Bio-Rad Laboratories, Hercules, CA). The samples were programmed to a temperature-step cycle at 95 °C for 10 minutes (min) initial denaturation and followed by forty (40) PCR cycles composed of denaturation at 95 °C for 30 seconds (sec), annealing temperature of 58 °C for 30 sec and extension at 72 °C for 30 sec as computed by the thermal cycler. After completion of the total PCR cycle, another 5-min extension at 72 °C followed. The samples were then subjected to a final holding temperature at 12 °C for 5 min. The PCR assay was performed three times.

Agarose gel electrophoresis and visualization. The samples were analyzed by performing electrophoresis in 1.8% agarose gel with 1x TAE (Tris-acetate-EDTA) as running buffer. The gel was immersed in ethidium bromide solution (10 mg/mL) for 15 min with constant agitation, visualized and photographed using the DigiDoc-It® Imaging System (UVP, USA) with attached documentation device (Canon®, USA).

Results

Blood samples were collected from a total of 246 apparently healthy cattle from five (5) randomly selected municipalities of Quirino Province, Philippines (Fig. 1). The bulk of the samples came from Diffun (77/246; 31.30%) and the least number of cattle was obtained from Cabarroguis (26/246; 10.57%) since Diffun and Cabarroguis had the greatest and the least cattle population in the province respectively (Table 1).

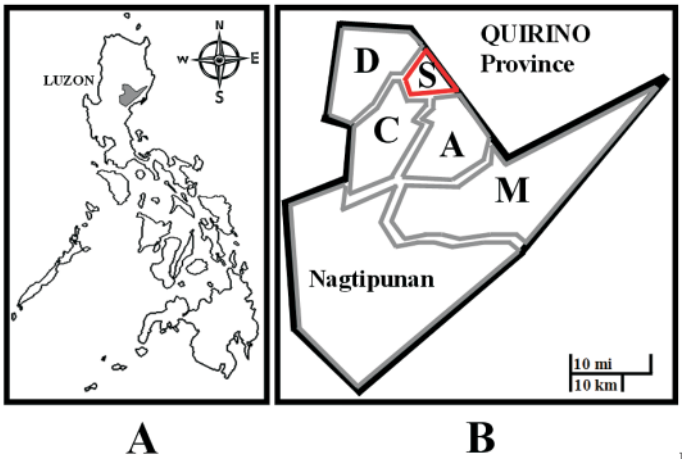


Fig. 1. Map of the sampling area of Quirino Province, Philippines. (A) General map of the Philippines showing the location of Quirino province (colored gray area) within Luzon. (B) The different municipalities used as sampling sites: Diffun- D; Cabarroguis- C; Saguday- S; Aglipay- A and Maddela- M. The red color-coded municipality (Saguday) represents the sampling area where *Trypanosoma evansi* positive cattle were recorded.

In terms of the age of the animals, the results demonstrated that the greater part of the sampled animals were more than three years old (96/246; 39.02%) whereas 10.57% (26/246) constitute the group of cattle with ages ranging from 2.1-3 years. In addition, based on the results, the cattle population was composed predominantly of females at 75.20% (Table 1).

Furthermore, the results of the physical examination using the nine-point scale body condition scoring system showed that BCS 3[Very thin, (107/246; 43.5%) had the highest frequency while BCS 1[Severely emaciated, (1/246; 0.40%) was the least encountered. Moreover, the data exhibited that the majority of the animals in Diffun and Saguday were thin (BCS 4) while most of the animals in Cabarroguis and Maddela and Aglipay were classified under BCS 3(Very thin) and BCS 2 (Emaciated) respectively (Table 1).

Table 1. Frequency and percentage distribution of (A) 246 blood samples according to sex, age, locality and BCS and (B*)* number of *Trypanosoma evansi* PCR positive blood samples in each study parameter

Parameters						A (%)	B
Sex							
Male						61 (24.80)	2
Female						185 (75.20)	1
Age (year)*							
≤1						88 (35.77)	0
1.1-2						36 (14.63)	1
2.1-3						26 (10.57)	1
>3						96 (39.02)	1
Localities (Total cattle population)							
(D) Diffun	(2,168)					77 (31.30)	0
(C) Cabarroguis	(719)					26 (10.57)	0
(S) Saguday	(1,184)					42 (17.07)	3
(A) Aglipay	(1,894)					68 (27.64)	0
(M) Maddela	(923)					33 (13.41)	0
Body condition scoring (BCS)**							
BCS	Locality						
	D	C	S	A	M		
1-Severely emaciated	0	1	0	0	0	1 (0.40)	0
2-Emaciated	1	1	1	35	8	46 (18.70)	0
3-Very thin	30	13	17	28	19	107 (43.50)	0
4-Thin	37	8	20	4	6	75 (30.50)	3
5-Moderate	9	3	4	1	0	17 (6.90)	0
6-Good	0	0	0	0	0	0	0
7-Very good	0	0	0	0	0	0	0
8-Obese	0	0	0	0	0	0	0
9-Very obese	0	0	0	0	0	0	0
Total	77	26	42	68	33		

*Age groups based on Philippine Recommendations for Beef Cattle Production (PCARRD, 1999). **Body condition scoring based on the report of WAGNER et al. (1988).

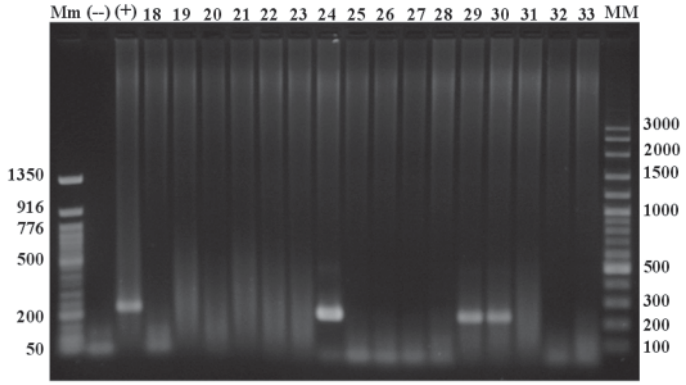
The polymerase chain reaction assay revealed that three animals were infected with *Trypanosoma evansi* (Fig. 2B). Two of the infected animals were males (2/61; 3.28%) and one was female (1/185; 0.54%). The infected animals came from varying age groups; one male from the 1.1-2 years group (1/36; 2.78%), another male from the 2.1-3 years group (1/26; 3.85%) and one female from the >3 years group (1/96; 1.04%). Consequently, all

the *Trypanosoma evansi* positive animals appeared thin with body condition scores of four. All the positive animals were from the municipality of Saguday, which was reported to have a municipal prevalence of 7.14% (3/42). In general, based on the PCR results, the provincial *T. evansi* prevalence of Quirino province was 1.22% (3/246) (Table 1).

Statistical analysis regarding the correlation between the study parameters (sex, age, BCS and locality) and the occurrence of parasite infection was determined to be no longer necessary due to the small number of PCR positive results signifying *Trypanosoma evansi* infected cattle.

Specificity	Primer Name	Sequence (5'-3')	Base pairs	Reference
T. evansi	PMURT TecF	5'-TGCAGACGACCTGACGCTACT-3'	21	Ravindran et al. (2008)
	PMURT TecR	5'-CTCCTAGAAGCTTCGGTCTCCT-3'	22	Njiru et al. (2004) Singh et al. (2004)

A



B

Fig. 2. Polymerase chain reaction detection of *Trypanosoma evansi* in cattle from Saguday. (A) *Trypanosoma evansi* species-specific primers (Invitrogen, Singapore). (B) PCR product analysis of samples from Saguday, Quirino Province by agarose gel electrophoresis. Mm [50 bp (New England Biolabs, USA)] and MM [100 bp (Vivantis Technologies, Malaysia)] molecular markers, Lane (--) and (+) are the negative (distilled water) and positive (*Trypanosoma evansi*;Tansui strain) controls respectively, Lane numbers (sample numbers) 24, 29 and 30 are samples from Saguday showing ~227 bp amplicons which are similar to the PCR band size of *T. evansi* positive control.

Discussion

The application of polymerase chain reaction assay for the species-specific detection of livestock trypanosome (*T. evansi*) in bovine blood samples from Quirino province, Philippines, was established in this research. Based on published national reports of the Bureau of Animal Industry (BAI), in Region 2 of the Philippines, surra cases were reported in the provinces of Quirino, Cagayan, Nueva Vizcaya and Isabela (ANONYMOUS, 2001; 2002). The present data constitute the first and only report of *T. evansi* infection in cattle in the province of Quirino. The data showed that three cattle (1.22%) out of 246 field blood samples from apparently healthy cattle collected from five municipalities of Quirino Province, Philippines, were found positive for *T. evansi* using PCR. All positive cases came from Saguday, which obtained a municipal prevalence of 7.14%.

Quirino is nearest the province of Nueva Vizcaya (~28 km) and farthest from Cagayan Valley (~193 km) among the Surra endemic provinces of Region 2. In 2001, the Bureau of Animal Industry published that at least one horse, cattle and carabao in the province of Cagayan and Isabela were infected by *T. evansi*. Furthermore, reports of *T. evansi* infection in cattle alone were documented in Nueva Vizcaya. Specifically in the province of Quirino, a few accounts of *T. evansi* infection were obtained from carabaos and horses but none was ever reported in cattle (ANONYMOUS, 2001). The findings suggest that *T. evansi* positive cattle likely acquired the infection from horses and carabaos in Quirino Province that were previously infected by the parasite.

It was observed that tabanid flies (*Tabanus* species) were present in the sampling areas throughout the duration of the sample collection. The identification of the vector host (tabanids) is considered a vital link in the positive *T. evansi* cases in Saguday. It is generally known that the distribution of arthropod-borne diseases is greatly associated with the presence and distribution of its vector host. The arthropods chiefly involved in the transmission of *Trypanosoma evansi* in the Philippines are the tabanid flies or horseflies (GARCIA and MANUEL, 1998). These are large, robust flies characterized by broad heads, bulging eyes and they are generally dark in color (WALL and SHEARER, 1997). There are two common species of tabanid flies in the country, namely *Tabanus striatus* and *T. reducens.* A previous study showed that the occurrence of trypanosomes is higher in *T. reducens* and was attributed to its greater size (GARCIA and MANUEL, 1998). Additionally, livestock trypanosomosis can also be transmitted mechanically by several blood-sucking flies (e.g stable fly and horn fly) (SOULSBY, 1982; BRUN et al., 1998; WATANAPOKASIN et al., 1998).

The success of trypanosome transmission from the infected animal host to another animal primarily relies on the interval between feedings of the arthropod fly vector. It has been documented that feeding time of at least five seconds was found to be sufficient to acquire and equally infect a host (LUCKINS, 1988). In principle, the shorter the feeding

interval, the higher the chances are to effect successful *T. evansi* transmission. This is because *T. evansi* does not undergo cyclical development in the fly vector and only has a limited life span in the proboscis of the fly. According to SOULSBY (1982), the survival time of *T. evansi* in the proboscis of a fly vector is only around 10-15 min. In addition to feeding intervals of the insect host, the increase in population of horseflies during monsoon season has been associated with the increased prevalence of surra.

The isolated cases of *T. evansi* infection confirmed by polymerase chain reaction are more likely attributed to the inaccessibility of the municipality of Saguday to the national road network, which thereby limits animal movement to other municipalities. Furthermore, as stated previously, the ability to transmit and infect cattle host successfully is largely dependent on the 10-15 minute window during which the parasite is viable in the proboscis of the fly (SOULSBY, 1982), therefore the inability of the vector to find a host within the period of infectivity maybe a plausible reason for the isolated cases. Accordingly, the breeds of cattle sampled were also determined since the existence of trypanosome resistant breeds such as N'Dama (ABENGA and VUZA, 2005) may account for the resistance of the cattle from other municipalities. However based on the history, all animals were Brahmans and these breeds are known to be susceptible to *T. evansi* infection.

Statistical analysis regarding the association of the study parameters (sex, age, body condition scoring and locality) and the positive molecular detection of the parasite infection in cattle was deemed no longer necessary due to the very small number of *Trypanosoma evansi* PCR positive results in Quirino Province. The data exhibited that none of the emaciated animal (BCS = 2) from Aglipay (35) and Maddela (8) and very thin (BCS = 3) cattle from Diffun (30), Cabarroguis (13), Saguday (17), Aglipay (28) and Maddela (19) proved to be positive for *Trypanosoma evansi* infection. Only three cattle from Saguday, which were relatively thin (BCS= 4), were found positive for *T. evansi* by polymerase chain reaction assay. There are no studies on cattle documenting the correlation of BCS and infection with *T. evansi*.

Reliable epidemiological research (e.g PCR-based study) is a precondition of designing effective control programs against trypanosomosis. It was recently advocated that accurate detection of trypanosomes in the host's blood is mostly dependent on the highly sensitive polymerase chain reaction assay (THUMBI et al., 2008). PCR allows detection of specific trypanosomal DNA through the use of species-specific primers. *T. evansi* primers used in the study amplified the expected amplicon size of ~227bp as previously described by MORLAIS et al. (1998). The display of approximately 227 base pair (bp) molecular bands, which was similar to the positive control (Fig. 2b), is identical to finding *T. evansi* in the blood. Similar band sizes were also obtained by several researchers using the same species-specific primers (MORLAIS et al., 1998; RAVINDRAN et al., 2008).

In a study conducted by MUGITTU et al. (2001), blood samples of 390 beef and dairy cattle were collected and 52 animals were found positive on both buffy coat and blood smear microscopy. Positive samples, as well as 62 randomly selected negative blood samples, were again tested with PCR using species-specific primers. Results revealed that 43% (27/62) of the negative samples yielded positive results. Likewise, a study conducted by RAVINDRAN et al. (2008) using PCR to examine blood samples from 61 camels and 44 donkeys in India exhibited a prevalence of (21/61) 34.40% and (3/44) 6.8% respectively. A provincial prevalence of 0.99% and 5.71% was recorded in cattle in the province of Angel Sandoval and German Busch, Bolivia after the blood samples were subjected to PCR assay (GONZALES et al., 2007).

Giemsa-stained blood smears from all the municipalities were found negative for *T. evansi* infection after parasitological examination (BPE). There are several factors that may contribute to this outcome. One of the important factors is the stage of parasite infection.

Trypanosomosis that can be encountered in the field can be in acute or chronic form. In the acute phase, the usual clinical signs in natural hosts are intermittent fever accompanied by accumulation of parasites in the blood (parasitemia) (THUMBI 2008; LUCKINS, 1998). However, as the disease progresses to the chronic phase the number of parasites in the blood disappears. The low levels of parasitemia prevent parasitological diagnosis of trypanosomes (PEREIRA DE ALMEIDA et al., 1998; SOULSBY, 1982).

Moreover, parasitological detection methods have only ~50% diagnostic sensitivity (NANTULYA, 1990). The study of PATHAK et al. (1997) also showed that microscopic examination of blood from camels infected with *T. evansi* failed to detect more than 50% of the infection. In addition, MASAKE et al. (2002), NANTULYA (1990) and DESQUESNES (2004) stated that detection of trypanosomes is unsuccessful when the numbers of parasites are too low, as is the case with chronic infections. This might be the probable reason why no trypanosomes were detected in the blood smear of 694 horses in a previous study (PAYNE et al., 1991).

In cases of reservoir hosts such as cattle and water buffaloes, the nature of the infection is subclinical (SOULSBY, 1982). Similarly, it is more likely that the infected animals in Saguday were in their subclinical stage of infection, since cattle are reservoir hosts. Therefore, there is less probability of detecting the parasites in the bloodstream by BPE. Moreover, the animals did not show any clinical signs which further indicates subclinical infection. In addition, according to GONZALES et al. (2003), it is but common to encounter an intermittent pattern of parasitaemia which are often very low in cases of *T. evansi* infection in cattle.

The difference in results demonstrated that PCR is a more sensitive and reliable method for the diagnosis of *T. evansi* infection than blood parasite examination. The results of the study validate the previous report that Region 2 of the Philippines has existing *T.*

evansi cases (MANUEL, 1998). In addition, based on the data obtained, the use of PCR for determining the prevalence of trypanosomosis in livestock is well recommended due to its high sensitivity and applicability for epidemiological purposes. The data also constitute the first report of *T. evansi* infection specifically in cattle from the province of Quirino. Lastly, the study presents the first molecular evidence of *Trypanosoma evansi* infection in cattle in the province of Quirino, Philippines.

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SAŽETAK

Trypanosoma evansi posvudašnji je protozoon koji invadira stoku na Filipinima. To je prvo izvješće o tripanosomozi goveda na području Quirino na temelju nalaza dobivenih lančanom reakcijom polimerazom. Ukupno je 246 uzoraka krvi bilo sakupljeno od naizgled zdravih goveda u pet općina na području Quirino. Prisutnost *T. evansi* dokazana u 3 (1,22%) životinje, u jedne ženke i dva mužjaka različite dobi (1,1-2 godine, 2,1-3 godine i više od 3 godine) u općini Saguday (3/42; prevalencija od 7,14%). U svih životinja bila je određena i tjelesna kondicija prema sustavu bodovanja od 1 do 9. Pregledom svih životinja ustanovljena su bila tri najčešća stupnja kondicije: 3 [vrlo mršava, (107/246; 43,50%)], 4 [mršava, (75/246; 30,50%)] i 2 [kahektična, (46/246; 18,70%)]. Goveda na području naselja Diffun i Saguday bila su mršava dok su goveda na području Cabarroguis, Maddela i Aglipay bila vrlo mršava i kahektična. Sve su pozitivne životinje u općini Saguday bile mršave. U goveda pozitivnih lančanom reakcijom polimerazom nisu bili dokazani paraziti u krvi. Rezultati ovog istraživanja predstavljaju i izvješće o prvom dokazu protozoona *Trypanosoma evansi* na području Quirino na Filipinima.

Ključne riječi: *Trypanosoma evansi*, sura, krvni parazit, lančana reakcija polimerazom, Quirino, Filipini