ORIGINAL PAPER

Isolation, cloning, and pathologic analysis of *Trypanosoma evansi* field isolates

**Hirohisa Mekata · Satoru Konnai · Claro N. Mingala ·
Nancy S. Abes · Charito A. Gutierrez ·
Alan P. Dargantes · William H. Witola · Noboru Inoue ·
Misao Onuma · Shiro Murata · Kazuhiko Ohashi**

Abstract In recent years, the emergence of highly pathogenic *Trypanosoma evansi* strains in the Philippines has resulted in substantial losses in livestock production. In this study, we isolated *T. evansi* from infected-water buffaloes in the Philippines and analyzed their virulence using mice and cattle. A total of 10 strains of *T. evansi* were isolated. Evaluation of the virulence of each strain using mice depicted significant differences among the strains in the prepatent period, the level of parasitemia, and the survival time of the infected animals. In mice infected with the highly pathogenic *T. evansi*, signs of excessive inflammation such as marked splenomegaly and increase more than 6-fold in the number of leukocytes were observed at 8 days post-infection. To study the virulence of the parasite strains in cattle (which are the common *T. evansi* hosts in Philippines), cattle were infected with the *T. evansi* isolates that showed high and low virulence in mice. The rate of parasite growth and the length of the prepatent periods were found to be similar to those observed in mice for the respective strains. The cattle infected with the highly pathogenic strain developed anemia and a marked decrease in leukocyte counts. To determine the cause of the pathological changes, we analyzed the expression levels of inflammatory cytokines and observed up-regulation of tumor necrosis factor-α in anemic infected cattle. Our findings suggest that the epidemic of *T. evansi* in the Philippines is characterized by *T. evansi* strains with varying virulences from low to very high pathogenicity in cattle.

H. Mekata · S. Konnai · M. Onuma · S. Murata · K. Ohashi (✉)
Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan
e-mail: okazu@vetmed.hokudai.ac.jp

C. N. Mingala · N. S. Abes · C. A. Gutierrez
Animal Health Laboratory, Philippine Carabao Center, Science City of Munoz, Nueva Ecija 3120, Philippines

A. P. Dargantes
College of Veterinary Medicine, Central Mindanao University, Musuan 8710 Bukidnon, Philippines

W. H. Witola
Department of Agricultural and Environmental Sciences, Tuskegee University, 312 Milbank Hall,
Tuskegee, AL 36088, USA

N. Inoue
National Research Centre for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan

Introduction

Trypanosoma evansi is a widely distributed flagellate protozoa that causes a disease called surra in domestic animals and is transmitted mechanically by biting flies such as *Tabanus* and *Stomoxys* spp. (Lun and Desser 1995; Reid 2002; Sumba et al. 1998; Otte and Abuabara 1991). The main clinical signs of *T. evansi* infection include fever, anemia, weight loss, lethargy, swelling of the hind limbs, and hemostatic abnormalities. Furthermore, *T. evansi* may exacerbate secondary infections and interfere with vaccinations because it suppresses the immunity of infected animals (Holland et al. 2003; Holland et al. 2001). In recent years, the damage caused by the parasite has been increasing due to expansion of the biting fly's range as well as transportation of infected livestock. Surra occurs in all 13 regions of the Philippines and has become a serious problem for the livestock industry. The Philippines government now regards surra as the second most important disease of livestock after fasciolosis and is planning to implement a national control program for surra (Reid 2002).

The pathogenicity of *T. evansi* varies significantly among strains and in different animal species (de Menezes et al. 2004; Queiroz et al. 2000). *T. evansi* infection is usually regarded as a serious disease of horses and camel, causing high mortality, whereas the disease is typically mild in other domestic animals (Luckins 1988; Silva et al. 1995). However, during the past decade, outbreaks with high mortality have occurred, not only in horses and camels, but also in buffaloes, cattle, and goats in the Philippines (Reid 2002). Although there are growing concerns about the appearance of highly pathogenic *T. evansi*, there are no reports about the virulence of the strains of *T. evansi* in the Philippines.

In contrast to human trypanosomiasis, which is caused by *Trypanosoma brucei* and *Trypanosoma cruzi*, the immuno-biological aspects of *T. evansi* infection have hardly been documented. The most reports have used rodents as the disease model, and not the originally infected host in the field, such as cattle and water buffaloes (Paim et al. 2011; Li et al. 2009; Baral et al. 2007; de Menezes et al. 2004). In *T. brucei* and *Trypanosoma congolense* infection, interferon-γ (IFN-γ) plays a major role in the control of infection (Hertz et al. 1998; Magez et al. 2006). However, overproduction of TNF could affect anemia (Baral et al. 2007).

In this study, we isolated *T. evansi* from infected water buffaloes in Luzon and Mindanao areas in the Philippines and analyzed the variations in pathogenicity among the isolates in mice and cattle. Further, we analyzed the expression patterns of inflammatory cytokines and other blood parameters in infected cattle.

Methods

Ethics statement

This study was carried out in strict accordance with the recommendations set out in the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science and the protocol for the animal experiments was approved by the Hokkaido University Animal Care and Use Committee (Approval number, 1023 and 11-0064, 1028 and 11-0060).

Isolation of field strains of *T. evansi*

From October 2008 to December 2009, we collected blood samples from *T. evansi*-infected water buffaloes in trypano-somiasis endemic areas of Luzon and Mindanao islands in the Philippines where there were many observed cases of water buffalo morbidity and mortality due to *T. evansi* infections. Blood samples were collected from the tail vein of infected water buffaloes and inoculated intraperitoneally in BALB/c mice.

All the inoculated mice were reared in a specific pathogen-free facility of the Philippine Carabao Center (Science City of Munoz, Philippines). Parasitemia in the inoculated mice was regularly monitored by collecting blood from the tail vein and analyzing by light microscopy. The mice with high parasitemia were anesthetized with isoflurane (Dainippon Sumitomo Pharma Co., Ltd, Osaka, Japan) and blood collected by cardiac puncture. A total of 22 *T. evansi* field isolates were obtained (Table 1).

Infections

All the BALB/c mice (male, 5- to 7-week-old) used in Japan were purchased from the Sankyo Labo Service Corporation, INC. (Tokyo, Japan). Those mice were maintained in a specific pathogen-free facility of the Graduate School of Veterinary Medicine, Hokkaido University.

All the cattle (*Bos tauras*, Holstein-Friesian, male 8- to 9-month-old) used in this study were obtained from the Experiment Farm, Field Science Center for Northern

Table 1 Details of parasite clones

Sampling site	Recognition number of buffalo	In vitro culture	Name of parasite clone
Luzon	2	+	L-2
	3	+	L-3
	10	ND[a]	
	13	+	L-13
	14	ND	
	16	+	L-16
	24	+	L-24
	26	ND	
	29	ND	
	33	ND	
	84	ND	
Mindanao	5	+	M-5
	8	ND	
	20	+	M-20
	28	ND	
	30	ND	
	31	ND	
	35	+	M-35
	36	+	M-36
	47	+	M-47
	57	ND	
	68	ND	

[a] No data

Biosphere, Hokkaido University (Sapporo, Japan). Those cattle were reared in the experimental animal facility of the Graduate School of Veterinary Medicine, Hokkaido University.

Axenic cultivation of *T. evansi*

The isolated *T. evansi* were cultivated in vitro by using a modified version of the procedure described in Hirumi and Hirumi (1989). The parasites were purified from the mice blood samples by diethylaminoethyl-cellulose chromatography (Whatman International Ltd., Maidstone, England) and transferred to 3 ml of modified Iscove's medium (HMI-9) and incubated at 37 °C in a 5 % CO_2 atmosphere (Lanham and Godfrey 1970). HMI-9 medium was prepared as previously described (Witola et al. 2004). In the initial stage of culture, the trypanosomes propagated slowly and thus, the cultures were passaged by diluting in the range of 1.0×10^5 to 1.0×10^6/ml. After 2–4 weeks, the trypanosomes began to propagate rapidly, and the cultures were from there on routinely diluted. The parasites that had adapted to axenic culture were cloned three times by limiting dilution. All the established cloned strains were cryopreserved at −80 °C.

The cloned parasites used for experimental infection were thawed and passaged in BALB/c mice by intraperitoneal injection. The parasites were then purified from the blood of parasitemic mice as described above.

Analysis of parasite virulence using mice

The virulence of 10 established strains were compared using BALB/c mice. The purified parasites (2.0×10^3) were

injected intraperitoneally into mice. The prepatent period (days before the first appearance of parasites in blood as observed by light microscopy), the level of parasitemia, and the survival time were recorded. The parasitemia levels were estimated by real-time PCR targeting *Rotat 1.2* in the parasite genome (Konnai et al. 2009). A standard curve for the analysis was set up using eight samples of 10-fold serially diluted *T. evansi* parasites. The number of leukocytes, erythrocytes and thrombocytes, the hemoglobin (Hb) concentration, and the hematocrit (Ht) were measured by an automated veterinary hematology analyzer, MEK-6450 Celltac α (Nihon Koden, Tokyo, Japan). The trypanosome strains were divided into different virulence categories depending on the median survival time of the infected mice (Table 2). Strains were considered to be of high and low virulence when the median survival time (Av.) + standard error (SE) was less than 12.86 days and when the median survival time Av. − SE was more than 14.56 days, respectively. The strains that did not fall the two categories described above were considered to be of moderate virulence.

Analysis of parasite virulence using cattle

The most virulent strain L2 and the least virulent strain L3 passaged in mice and purified as described were injected (2.0×10^7) subcutaneously into cattle's cervical region. The body temperature of the infected cattle was monitored, and jugular blood was collected for determination of parasitemia and blood parameters as described above.
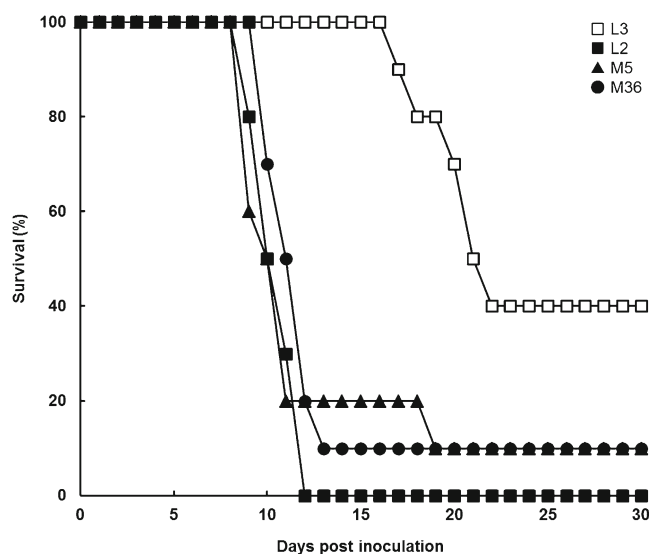
Table 2 Analysis of the virulence of *T. evansi* field isolates using mice

Name of parasite	Prepatent period[a]	Survival time[a]	Virulence[b]
L-2	6.1±0.23	10.6±0.37	High
L-3	10.9±1.57	19.8±1.12	Low
L-13	6.3±0.33	14.3±0.88	Moderate
L-16	8.3±2.59	13.8±3.77	Moderate
L-24	5.3±0.33	13.3±2.40	Moderate
M-5	6.2±1.12	10.9±1.06	High
M-20	6.6±0.68	14.0±1.45	Moderate
M-35	5.4±0.24	13.5±1.04	Moderate
M-36	6.1±0.20	11.2±0.36	High
M-47	5.7±0.30	15.8±1.24	Moderate
Av.	6.7±0.53	13.7±0.86	

[a] Average (Av.) ± standard error (SE)

[b] Strains were considered to be of high and low virulence when the Av. + SE was less than 12.86 days and when the Av. − SE was more than 14.56 days, respectively. The strains that did not fall the two categories described above were considered to be of moderate virulence

Fig. 1 The survival curve of high and low pathogenic strain-infected mice. The Kaplan–Maier method was used to construct curves for survival. The prepatent period and the mean survival time are shown in Table 2. The log rank test was used to compare the differences between low pathogenic strain (*L3*) infected group and highly pathogenic strain (*L2*, *M5*, and *M36*) infected groups. $p<0.01$ (L2), $p<0.05$ (M5), and $p=0.01$ (M36)

Table 3 Mice parasitemia on day 6 and 8 post-inoculation

Post inoculation	Name of strain	Virulence	Recognition number	Parasitemia (/ml)
Day 6	L-3	Low	Ba6-1	$<10^4$
			Ba6-2	$<10^4$
			Ba6-3	$<10^4$
Day 6	L-2	High	Ba6-4	1.96×10^7
			Ba6-5	5.83×10^7
			Ba6-6	5.35×10^7
Day 6	M-5	High	Ba6-7	4.42×10^7
			Ba6-8	5.56×10^7
			Ba6-9	6.05×10^6
Day 8	L-3	Low	Ba8-1	4.49×10^5
			Ba8-2	$<10^4$
			Ba8-3	1.45×10^5
Day 8	L-2	High	Ba8-4	1.98×10^8
			Ba8-5	1.96×10^8
			Ba8-6	1.98×10^8
Day 8	M-5	High	Ba8-7	2.35×10^8
			Ba8-8	2.38×10^8

Quantitative real-time PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA). After treatment with DNase I (Invitrogen) to remove residual DNA, reverse transcription was performed using the reverse transcriptase M-MLV (TaKaRa, Otsu, Japan) according to the protocol supplied by TaKaRa. Quantitative real-time PCR analyses were performed using the SYBR® Premix DimerEraser™ (TaKaRa) and a real-time PCR system (LightCycler® 480 Real-Time PCR System; Roche Applied Science, Indianapolis, IN) following the manufacturer's instructions. Primer3Plus was used to design the primer sets used. "2nd Derivative Max" was used to determine the value of crossing point. Glucuronidase, beta (*GUSB*), and heat shock protein 90 (*HSP90*) were used as internal control genes. Relative amounts were calculated using the comparative threshold cycle method (Livak and Schmittgen 2001). Confirmation of the specificity of the PCR products was done by performing amplicon melting-curve analysis and agarose gel electrophoresis.

Statistical analysis

The Statcel3 software (The publisher OMS Ltd., Tokorozawa, Japan) was used for all the statistical analyses. Data are presented as mean ± standard error (SE). The two-tailed Student's *t* test was used to compare differences between two groups. The Log rank test was used for survival analysis (Mantel 1966; Peto 1972). In this study, $p < 0.05$ was considered significant.

Results

Establishment of parasite clones

The establishment of parasite clones is indispensable for analysis of the virulence. Therefore, clones were established from the field isolates of *T. evansi* by limiting dilution. A total of 10 clones of trypanosomes were established from 22 field isolates and designated with the name M or L (initial of the island where sample came from) and the recognition number for the water buffalo from which the strain was isolated (Table 1). From a morphological point of view, no differences were observed among the clones.

Analysis of parasite virulence using mice

The 10 cloned strains of *T. evansi* were equally injected into groups of mice. Based on the median survival time (13.72± 0.86), three cloned strains (L-2, M-5, and M-36) were classified as highly virulent, six cloned strains (L-13, L-16, L-24, M-20, M-35, and M-47) were considered as moderately virulent, and the remaining one cloned strain (L3) had low virulence in mice (Table 2). The prepatent periods were

Table 4 Infected animals' blood parameter on day 6 post-inoculation

Name of strain (Virulence)	No. of WBC ($\times 10^2$/ml)	No. of RBC ($\times 10^4$/ml)	Hemoglobin (g/dl)	Hematocrit (%)	No. of thrombocyte ($\times 10^4$/ml)
L-3 (low)	42±8	820±11	13.2±0.3	37.2±0.1	66.5±11.4
L-2 (high)	290±50	800±16	12.2±0.2	34.6±0.5**	11.8±3.4*
M-5 (high)	484±58	694±24*	9.6±0.3**	29.9±0.8**	37.1±31.1

Results were shown in Av. ± SE. Student *t* test were performed between the mice infected with high virulence and the low virulence

*$p < 0.05$; **$p < 0.01$

Fig. 2 The spleens of infected mice at day 8 post-infection. Mice were inoculated intraperitoneally with 2.0×10^3 *T. evansi* strain L2, L3, and M5. Each representative spleen at day 8 post-infection is shown
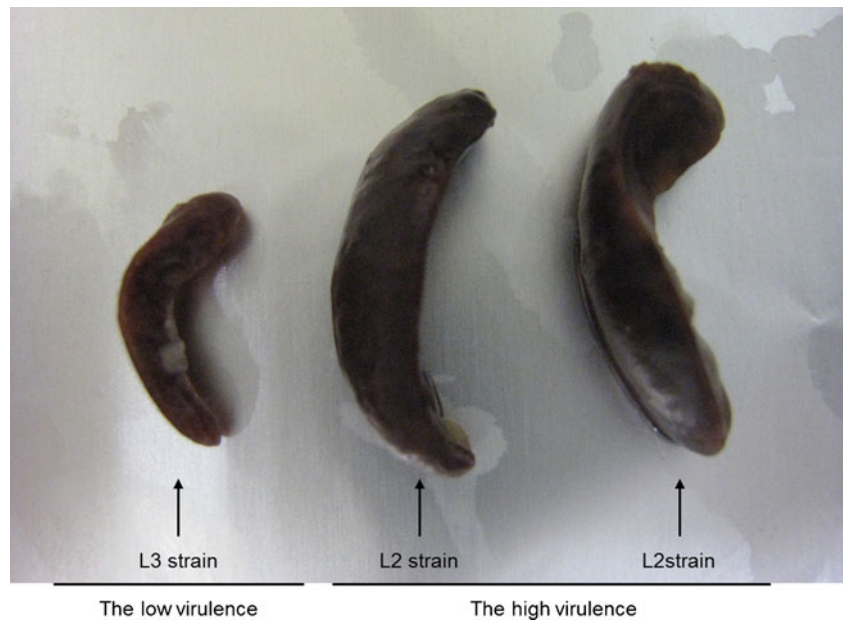
significantly shorter for *T. evansi* strains belonging to the high virulence category compared to strains of the low virulence category (L-2, *p*=0.0074; M-5, *p*=0.029; M-36, *p*=0.010). The survival curve for the high and low virulence strains is shown in Fig. 1. The quantitative analyses of parasitemia were performed in the mice infected with *T. evansi* L3 strain belonging to the low virulence category and L2 and M5 strains belonging to the high virulence category. In the mice infected with the low virulence category, the parasitemia was less than 10^4/ml blood on day 6 p.i. and less than 10^6/ml blood on day 8 p.i. (Table 3). On the other hand, in the mice infected with the high virulence categories, the parasitemias were more than 10^6/ml blood on day 6 p.i. and more than 10^8/ml blood on day 8 p.i. (Table 3). The blood parameter analyses were performed. In the mice infected with the high virulence categories the number of leukocytes on day 6 p.i. was 6.9–11.5-fold more than in those infected with the low virulence
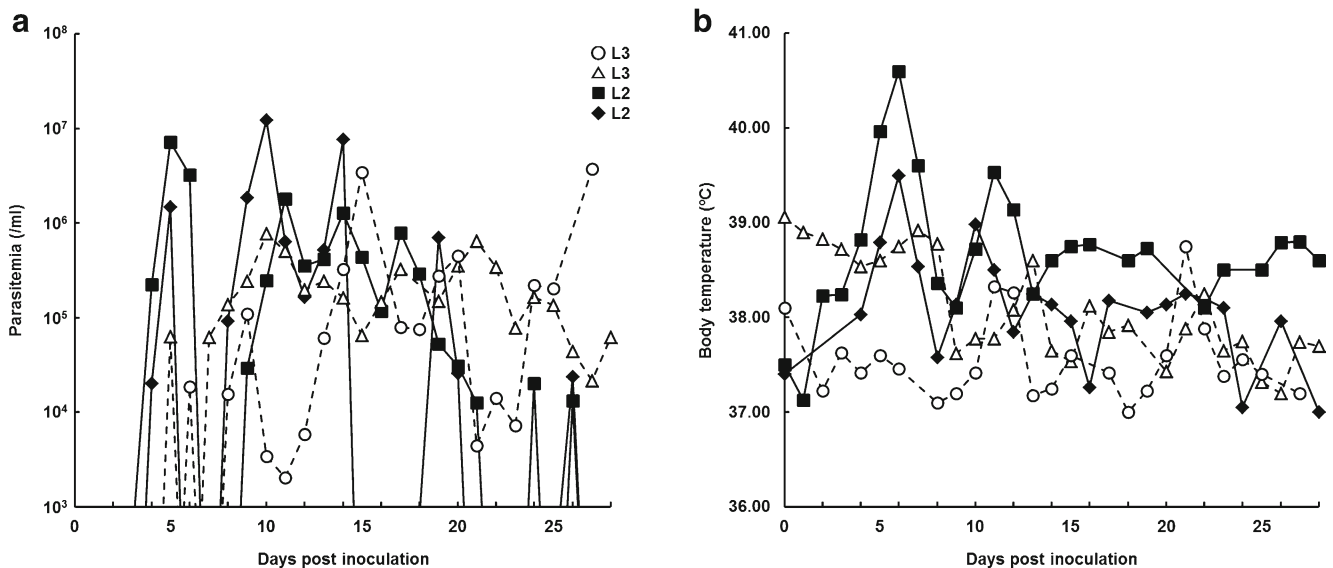

Fig. 3 The changes in parasitemia and body temperature in infected cattle. Cattle were inoculated subcutaneously with 2.0×10^7 *T. evansi* strain L2 and L3. The changes in parasitemia (**a**) and body temperature (**b**) are shown
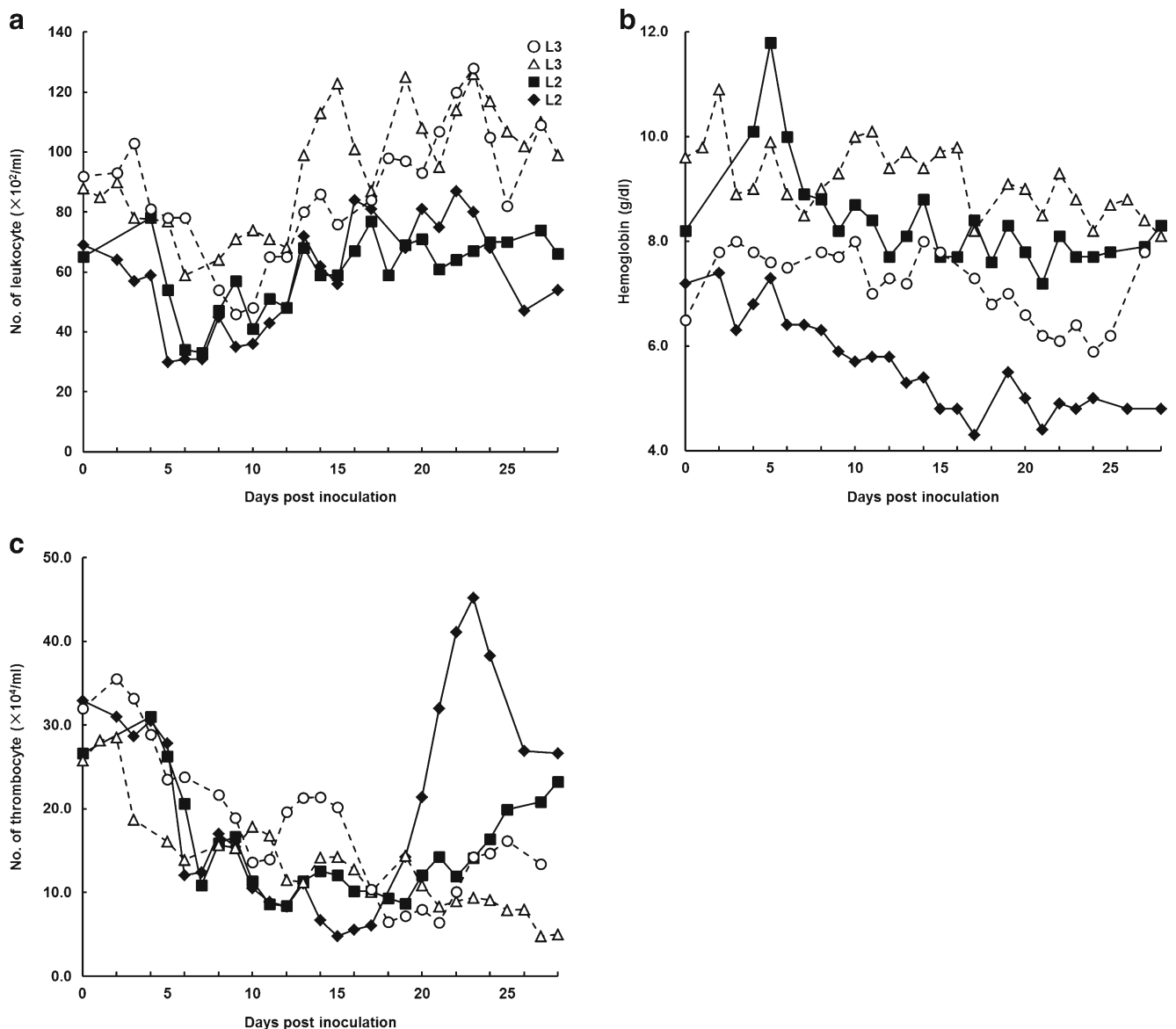
Fig. 4 The blood parameters of *T. evansi*-infected cattle. The changes in the **a** number of leukocytes, **b** hemoglobin concentration, and **c** thrombocyte count in infected cattle. Cattle were inoculated subcutaneously with 2.0×10^7 *T. evansi* strain L2 and L3

categories (Table 4). In addition, the number of erythrocytes, the hemoglobin concentration, and the hematocrit were decreased and anemia was observed in the mice infected with the high virulence categories (Table 4). Furthermore, splenomegalies associated with severe inflammation were observed in mice infected with the high virulence categories (Fig. 2).

Analysis of parasite virulence using cattle

To determine whether the parasite virulence observed in mice corresponded to cattle, we analyzed the pathogenicity of the most virulent (L2) and the least virulent (L3) strains

of *T. evansi* using cattle. Although the parasitemic waves were observed, the high parasitemia ($>10^6$/ml) was observed only at four different days of total 28 days of infection with the most virulent strain (Fig. 3a). On the other hand, no high parasitemia was observed in cattle infected with the low virulence strain (Fig. 3a). Furthermore, the cattle infected with the high virulence strain showed fever of more than 39 °C (Fig. 3b) while cattle infected with the low virulence did not show any fever (Fig. 3b). The number of leukocytes was markedly decreased, particularly in cattle infected with the high virulence strain (Fig. 4a). One of the cattle infected with the high virulence strain showed severe anemia. However, no cattle infected with the low virulence any
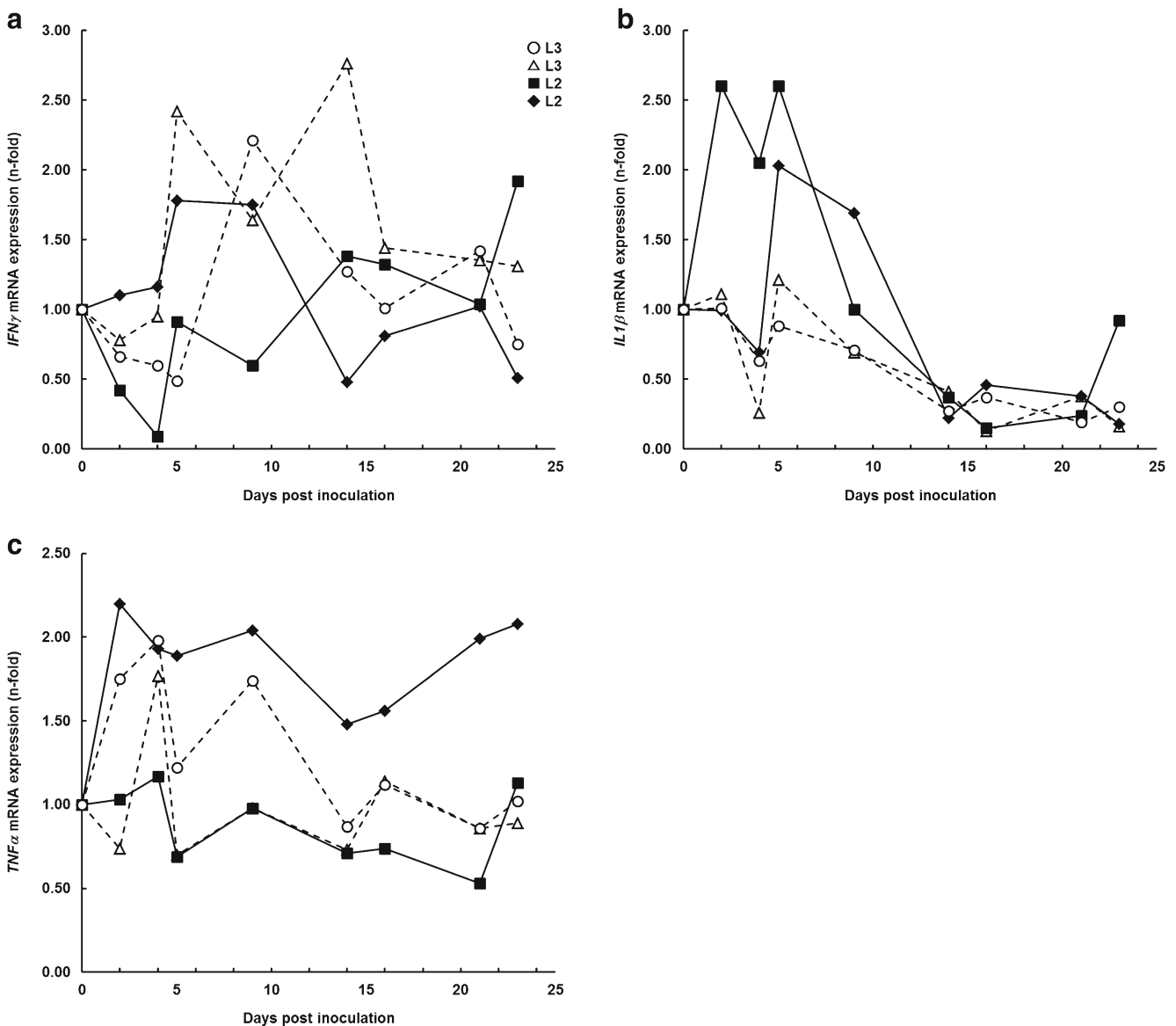
Fig. 5 The expression levels of inflammatory cytokines in *T. evansi*-infected cattle. The expression levels of *IFNγ* (**a**), *IL1β* (**b**), and *TNFα* (**c**) in the peripheral blood at 2, 4, 5, 9, 14, 16, 21 and 23 days post-infection

symptoms suggestive of anemia (Fig. 4b). As regards the number of thrombocytes, cattle infected with all the *T. evansi* strains showed a marked decrease (Fig. 4c).

Comparison of cytokine expression in infected cattle

To analyze the immune responses in cattle infected with *T. evansi*, the kinetics of inflammatory cytokines expression (IFNγ, IL1β, and TNFα) in the peripheral blood were analyzed. The expression of *IFNγ* was up-regulated in cattle infected with the low virulence strain (Fig. 5a). The expression of *IL1β* was up-regulated in the early phase of infection and down-regulated in the late phase (Fig. 5b). As regards

the expression of *TNFα*, high expression was observed in cattle that showed anemia (Fig. 5c).

Discussion

The aim of this study was to analyze the virulence of *T. evansi* field isolates. Therefore, we cloned the field isolates of *T. evansi* from infected water buffaloes and performed virulence assessment tests using mice and cattle. There have been some reports about the virulence assessment of field isolates of trypanosomes using rodents or ruminant (de Menezes et al. 2004; Holzmuller et al. 2008; Masumu et

al. 2006; Onah et al. 1999; Onah et al. 1996). However, there have been few reports about the virulence assessment tests using both livestock and rodents.

A total of 10 cloned strains of *T. evansi* were analyzed for virulence using mice. Three strains were considered as highly virulent, six strains were moderately virulent, and one strain had low virulence. Significant differences of the mean survival time, the prepatent period, and the parasitemia on days 6 and 8 post-infection were observed between the high and low virulence strain in mice. However, in vitro culture, the parasite growth rate was not different among the strains (data not shown). The difference in the parasite growth rate in vivo among the strains could be attributed to the easy with which the highly virulent strains establish infection compared to the low virulence strains. In the mice infected with the highly virulent strains, significant increases in the number of leukocytes (6.9–11.5-fold) and splenomegalies were observed on day 8 post infection. However, the severity of anemia was mild. Based on these findings, it can be postulated that the pathology associated with acute *T. evansi* infection results from severe inflammation attributable to cytokine storm as is the case in cerebral malaria (Erdman et al. 2008).

The difference in parasite growth between the high and low virulent strains observed in mice was consistent in cattle. Furthermore, a marked decrease in the number of leukocytes as well as hyperthermia and anemia were observed in cattle infected with highly pathogenic strains. These results suggest that different epidemics of surra in the Philippines are characterized based on the strain involved (whether of high or low virulence) and, therefore, control strategies should be planned based on the ravaging strain.

In this study, we attempted to simulate natural infection in cattle by inoculating parasites subcutaneously. This subcutaneous route of inoculation of the parasites in cattle, compared to the intraperitoneal route in mice, cattle could have contributed to decreased disease symptoms in cattle compared to those observed in mice. Wei et al. have reported that the intradermal infections with trypanosome resulted in effective activation of the innate immunity which made the host animal to be a 100 times more resistant to infection than in intraperitoneal infections (Wei et al. 2011). Furthermore, cattle are regarded to be less susceptible to *T. evansi* than water buffalo or horse. Therefore, for a comprehensive assessment of virulence, the inoculation method and the animal species used should be considered.

In this study, marked decreases in the number of leukocytes were observed in cattle infected with highly virulent strains. There have been some reports about immunosuppression associated with *T. evansi* infection (Holland et al. 2003; Holland et al. 2001). Therefore, mixed infection with

other pathogens such as *Pasteurella* could cause exacerbate infection under field conditions. Future studies are needed to elucidate the immunosuppression associated with the highly virulent *T. evansi* strain infection.

In this study, the expression analyses of *IFNγ*, *IL1β*, and *TNFα* in *T. evansi*-infected cattle were performed. Inflammatory cytokines such as IFN-γ or TNF-α contribute to the protection against infection (Magez et al. 1997). However, inflammatory responses resulting from the overproduction of these cytokines could deleterious to the host (Masocha et al. 2004; Magez et al. 1999; Magez et al. 2004). Significant up-regulation of *IFNγ* or *IL1β* expression which could contribute to excessive inflammation was not observed during the infection period. On the other hand, severe anemia and significant up-regulation of *TNFα* were observed in the cow infected with the highly virulent strain, suggesting a relationship between *TNFα* expressing and anemia. This relationship between TNF-α production and anemia has been reported in *T. congolense* and *Trypanosoma vivax* infections (Sileghem et al. 1994).

In this study, we confirmed the presence of high virulence *T. evansi* in Philippines which propagates effectively in the host animals. However, the factors underlying this strain virulence are yet to be elucidated. Hereafter, more detailed pathogenesis analyses for field *T. evansi* isolates are required.

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