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# Pathogenic Effects and Prepatent Periods of *Trypanosoma evansi* Isolates from Indonesia in Mice

(Kesan Patogen dan Tempoh Prapaten Pengasingan *Trypanosoma evansi* pada Mencit dari Indonesia)

Dyah H. Sawitri[1], April H. Wardhana[1,2], Mohamad Sadikin[3], Heri Wibowo[4] & Marc Desquesnes[5]

[1]*Indonesian Research Centre for Veterinary Science, Bogor, Indonesia 16114*
[2]*Faculty of Veterinary Medicine, University of Airlangga, Surabaya, Indonesia*
[3]*Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia*
[4]*Department of Parasitology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia*
[5]*InterTryp, Université Montpellier, CIRAD, IRD, F-34398 Montpellier, France*

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ABSTRACT

*Trypanosoma evansi* is an extracellular blood protozoan parasite that causes Surra in livestock, but only a few studies have been conducted on the pathogenicity of its isolates. Meanwhile, it was discovered that the rate of parasite growth and the length of the prepatent periods were equivalent in different strains of cattle and mice. Thus, further research into the pathogenic effects and pre-patent periods of *T. evansi* in mice is necessary to aid in the epidemiological understanding and field treatment strategy for Surra. The purpose of this study was to ascertain the pathogenic potential of *T. evansi* isolates from Indonesia and their prepatent periods. A total of 32 *T. evansi* isolates from various regions in Indonesia were inoculated intraperitoneally into three male DDY mice ($10^4$ parasites/0.3 mL) for each isolate. Additionally, the parasitaemia level and mortality of the mice were determined every 2 days, and the data were analysed using ANOVA with a 95% confidence level. The results indicated that *T. evansi* isolates exhibited virulence levels classified as high, moderate and low. Each virulence exhibited a distinct parasitaemia pattern. However, high virulence was not associated with short prepatent periods. The results indicated that the surra epidemic in Indonesia is characterised by strains with varying degree of virulence in water buffaloes necessitating the development of effective treatment strategies.

Keywords: Mice; pathogenic; prepatent; *Trypanosoma evansi*; virulence

ABSTRAK

*Trypanosoma evansi* ialah parasit protozoa darah ekstrasel yang menyebabkan Surra dalam ternakan, tetapi hanya beberapa kajian telah dijalankan tentang kepatogenan pencilannya. Sementara itu, kadar pertumbuhan parasit dan panjang tempoh prapaten dalam pelbagai jenis lembu dan mencit didapati adalah setara. Oleh itu, penyelidikan lanjut mengenai kesan kepatogenan dan tempoh pra-paten *T. evansi* dalam mencit adalah perlu untuk membantu dalam pemahaman epidemiologi dan strategi rawatan lapangan untuk Surra. Tujuan kajian ini adalah untuk menentukan potensi patogen bagi pencilan *T. evansi* dari Indonesia dan tempoh prapatennya. Sebanyak 32 pencilan *T. evansi* dari pelbagai wilayah di Indonesia telah disuntik secara intraperitoneal ke dalam tiga mencit DDY jantan ($10^4$ parasit/0.3 mL) bagi setiap pencilan. Di samping itu, tahap parasitemia dan kematian mencit ditentukan setiap 2 hari dan data dianalisis menggunakan ANOVA dengan tahap keyakinan 95%. Hasil kajian ini menunjukkan bahawa pencilan *T. evansi* menunjukkan tahap kevirulenan yang dikelaskan sebagai tinggi, sederhana dan rendah. Setiap kevirulenan mempamerkan corak parasitemia yang berbeza. Namun begitu, kevirulenan yang tinggi tidak dikaitkan dengan tempoh prapaten yang singkat. Hasil kajian menunjukkan bahawa wabak surra di Indonesia dicirikan oleh strain dengan pelbagai tahap kevirulenan dalam kerbau air yang memerlukan pembangunan strategi rawatan yang berkesan.

Kata kunci: Kevirulenan; mencit; patogen; prapaten; *Trypanosoma evansi*

## Introduction

Surra is a parasitic disease caused by *Trypanosoma evansi*, a blood protozoan parasite that circulates in mammalian extracellular fluid. The parasite is mechanically transmitted in South East Asia by bloodsucking flies, primarily *Tabanus rubidus*, *T. striatus*, *Stomoxys calcitrans* and *Chrysops* spp. (Desquesnes et al. 2013a). It is a major problem for livestock and results in significant economic losses due to abortion in late pregnancy, delayed oestrus, decreased body weight gains, immune suppression and death (Desquesnes et al. 2013b; Jittapalapong et al. 2009; Sengupta et al. 2012). Surra caused by *T. evansi* was first reported in Indonesia in 1897, when it infected a herd of horses in Semarang, Central Java. It then spread rapidly throughout the Indonesian archipelago, aided by the abundance of haematophagous flies and the lack of surra treatment drugs at the time. Surra prevalence in cattle, horses and water buffaloes ranged between 0.28 and 69.3%, between 2010 and 2014, according to four Disease Investigation Centers (DIC) in Indonesia namely DIC Wates (Yogyakarta), DIC Subang (West Java), DIC Bukit Tinggi (West Sumatra) and DIC Denpasar (Bali). Until 2009, only Sumba Island was reported as being free of *T. evansi* in Indonesia. However, a surra outbreak occurred there in 2010 during a traditional horse race. Over 4,268 livestock were infected, resulting in 1,760 livestock deaths, including 1,159 horses, 600 water buffaloes and 1 head of cattle. Economic losses grew from $4.22 million in 2010 to $16.72 million in 2012 (Department of Animal Husbandry District of East Sumba report, unpublished data). In 2013 to 2014, a second multifocal surra outbreak occurred in Banten province (West Java), followed by outbreaks in several other regions of Sumatra Island. Another surra outbreak occurred in 2016 in Bogor, Indonesia, involving water buffaloes and dairy cattle (West Java). The Indonesian government places surra on a list of strategic contagious animal diseases as a result of the severe financial losses.

In comparison to other trypanosomes, *T. evansi* is the most prevalent pathogenic trypanosome in tropical and subtropical areas (Desquesnes 2004). Surra clinical signs vary in most domestic and many wild animals depending on the level of *T. evansi* virulence, host species, general stress placed on the hosts and local epidemiological characteristics (Hoare 1972). Regardless of the biological differences in their pathogenic effects on the host, *T. evansi* infection generally results in immunosuppression, cachexia and anemia (Desquesnes et al. 2013b). Magez et al. (2020) claimed that trypanosomes have discovered a way to evade antibody-mediated death by inhibiting the complement system. As a result of this mechanism, innate immune control of infection is critical for host-parasite interaction, infection susceptibility and parasitaemia control.

In Africa, it is believed that this parasite is more pathogenic to camels and horses than to cattle (Dia & Desquesnes 2007). Similar reports in Latin America indicated that surra is more prevalent and critical in horses, dogs, and buffaloes, but is less prevalent and pathogenic in cattle (Desquesnes 2004). Diverse observations are made in Asia, where *T. evansi* is a significant pathogen in cattle, water buffaloes, horses and pigs (Holland et al. 2005). Meanwhile, surra cases primarily involve camels in Saudi Arabia, Sudan, Kenya, Spain and mainland France (Desquesnes et al. 2008; Gutierrez et al. 2010; Salim et al. 2011). According to a report by the Directorate General Livestock Services (DGLS), horses are the most sensitive animals, followed by water buffaloes and cattle in Indonesia (Ditjennak 2012).

The susceptibility to trypanosome infection varies between inbred mouse strains as well. BALB/c mice are more susceptible, whereas C57Bl/6 mice are more resistant, as measured by immunosuppression and survival time (Ogunremi & Tabel 1995; Tabel, Kaushik & Uzonna 2000). Six strains of mice (Balb/c, DPJ, CBA/CAH, C57BL/6J, ARC and Quackenbush) are, however, equally susceptible to *T. evansi* (Reid & Husein 1998). According to Sawitri, Wardhana and Wibowo (2017), DDY mouse strain is susceptible to *T. evansi*. Mekata et al. (2013), on the other hand, stated that high and low virulent strains of *T. evansi* in cattle demonstrated comparable and consistent parasite multiplication rates and prepatent periods in mice. This statement is supported by other reports made using rodents as model to study surra in cattle and water buffaloes (Baral et al. 2007; De Menezes et al. 2004; Paim et al. 2011). Although there is a growing concern about the emergence of highly pathogenic *T. evansi*, the study of virulence of *T. evansi* stocks from Indonesia is very limited. Such studies are fundamental to better understand and control surra in Indonesia, the largest archipelago country. Over the last decade, surra outbreaks with high mortality rate have occurred in Indonesia, not only in horses but also in water buffaloes and cattle (Ditjennak 2012). Variable levels of pathogenic expression of the disease have been discovered in different regions, indicating that the disease has a low impact in some areas but a high impact in others. Additionally, it is possible that even within a region, *T. evansi* virulence may vary among strains. The purpose of this study was to assess the virulence of *T. evansi*

stocks collected among water buffaloes from various geographical origins in experimentally infected mice.

## Materials and Methods

### *Trypanosoma evansi* stocks and field isolates

All isolates were originally obtained from water buffaloes. Thirty-two *T. evansi* isolates were used in this study (Table 1). Sixteen *T. evansi* isolates were collected in the field (circulating isolates) in East Sumba, Pandeglang, Lebak and Bogor (2012 to 2014). The remaining 16 isolates were liquid nitrogen-preserved (cryopreserved) stock isolates from the BBLitvet Culture Collection (BCC-Bogor, Indonesia). For the field isolates, blood samples were collected from the jugular vein of infected water buffaloes and inoculated intraperitoneally in DDY mice. When parasitaemia reached $10^7$ parasites/mL of blood, mice were anesthetised, and blood was collected via cardiac puncture. The blood was then mixed with PBS supplemented with 1% glucose and 10% glycerol to be stored cryopreserved in BBC until use.

TABLE 1. List of *T. evansi* isolates collected from water buffaloes and were used in this study

| No. | Isolate ID | BCC code | Isolate Origin (subdistrict, district, province) | Year of isolation | Status |
|---|---|---|---|---|---|
| 1 | Bang 87 | P0176 | Bangkalan, Bangkalan, East Java | 1988 | Stock/out |
| 2 | Bang 85 | P0162 | Burneh,Bangkalan, East Java | 1988 | Stock/out* |
| 3 | Pml 287 | P0232 | Pemalang, Pemalang, Central Java | 1996 | Stock/end** |
| 4 | Pml 291 | P0233 | Taman, Pemalang, Central Java | 1997 | Stock/end |
| 5 | Sbw 340 | P0202 | Lape Lopok, Sumbawa Besar, West Nusa Tenggara | 1998 | Stock/out |
| 6 | Sbw 341 | P0203 | Lape Lopok, Sumbawa Besar, West Nusa Tenggara | 1998 | Stock/out |
| 7 | Sbw 363 | P0213 | Lape Lopok, Sumbawa Besar, West Nusa Tenggara | 1999 | Stock/out |
| 8 | Sbw 364 | P030 | Lape Lopok, Sumbawa Besar, West Nusa Tenggara | 1999 | Stock/out |
| 9 | Sbw 366 | P029 | Lape Lopok, Sumbawa Besar, West Nusa Tenggara | 1999 | Stock/out |
| 10 | Smi 68 | P0169 | Surade,Sukabumi, West Java | 1985 | Stock/end |
| 11 | Smi 369 | P0125 | Surade, Sukabumi, West Java | 2008 | Stock/end |
| 12 | Bwi 218 | P075 | Kalibaru, Banyuwangi, East Java | 1992 | Stock/end |
| 13 | Bwi 222 | P076 | Kalibaru, Banyuwangi, East Java | 1992 | Stock/end |
| 14 | Bwi 228 | P077 | Kalibaru, Banyuwangi, East Java | 1992 | Stock/end |
| 15 | Ash129 | P0192 | Tanjung Muda, Asahan, North Sumatera | 1990 | Stock/end |
| 16 | Ash 133 | P0178 | Limapuluh, Asahan, Nort Sumatera | 1990 | Stock/end |
| 17 | Smb 370 | P0206 | Wajelo, Sumba Timur, East Nusa Tenggara | 2012 | Circ***/Out |
| 18 | Smb 371 | P0207 | Wajelo, Sumba Timur, East Nusa Tenggara | 2012 | Circ/Out |
| 19 | Smb 372 | P0208 | Wajelo, Sumba Timur, East Nusa Tenggara | 2012 | Circ/Out |
| 20 | Smb 373 | P0209 | Wajelo, Sumba Timur, East Nusa Tenggara | 2012 | Circ/Out |
| 21 | Smb 374 | P0210 | Wajelo, Sumba Timur, East Nusa Tenggara | 2012 | Circ/Out |
| 22 | Smb 375 | P0211 | Wajelo, Sumba Timur, East Nusa Tenggara | 2012 | Circ/Out |
| 23 | Lbk 376 | P0215 | Lebak, Banten | 2013 | Circ/Out |
| 24 | Bgr 377 | P0216 | Cijeruk, Bogor, West Java | 2013 | Circ/Out |
| 25 | Pdg 378 | P0217 | Cisata, Pandeglang, Banten | 2013 | Circ/Out |
| 26 | Pdg 379 | P0218 | Cisata, Pandeglang, Banten | 2013 | Circ/Out |
| 27 | Pdg 380 | P0219 | Cisata, Pandeglang, Banten | 2013 | Circ/Out |
| 28 | Pdg 381 | P0220 | Cisata, Pandeglang, Banten | 2013 | Circ/Out |
| 29 | Pdg 382 | P0221 | Carita, Pandeglang, Banten | 2014 | Circ/Out |
| 30 | Pdg 384 | P0223 | Carita, Pandeglang, Banten | 2014 | Circ/Out |
| 31 | Pdg 386 | P0225 | Cisata, Pandeglang, Banten | 2014 | Circ/Out |
| 32 | Pdg 388 | P0227 | Cisata, Pandeglang, Banten | 2014 | Circ/Out |

*Out =outbreak *** End =endemic ***Circ=circulating

Additionally, all *T. evansi* stocks preserved into liquid nitrogen were thawed before being intraperitoneally inoculated into DDY mice. Parasitaemia was regularly monitored every 2 days via blood collection from the tail vein and light microscopic observation. Mice with high parasitaemia ($10^7$ parasites/mL of blood) were anesthetised and blood was collected using a microsyringe via intracardiac puncture. Furthermore, the parasites were inoculated into groups of experimental mice. The research was conducted at the Indonesian Research Centre for Veterinary Science in Bogor, Indonesia. The protocol of this study was approved by the Ethics Committee of the Faculty of Medicine, University of Indonesia in Jakarta, Indonesia (124/H2.F1/ETIK/2013).

Stock isolates and circulating isolates collected from outbreak and endemic areas between 1985 and 2008 and 2012 to 2014, respectively. All *T. evansi* isolates were stored at the BBlitvet Culture Collection (BCC), in Bogor, Indonesia.

### PARASITE VIRULENCE IN MICE

In this study, 96 DDY mice (male, 10-12 weeks of age) with an average body weight of 20 to 25 g were used. After a 15-day adaptation period, the animals were divided into 32 groups of three mice each. Intraperitoneally, animals from groups K1–K32 were inoculated with 0.3 mL of blood containing $10^4$ *T. evansi* from each isolate. The same route was used to administer PBS-glucose solution to animals in group N (negative controls). Every 2 days, mice were observed, and blood was collected. As indicated below, blood was collected from the tail and examined under a light microscope (× 400).

According to the median survival time of infected mice, trypanosome stocks were classified into different virulence categories. For 4 weeks, until the mice died, the prepatent period (day of first detection of parasites in blood), parasitaemia level, and survival time were recorded.

### DETERMINATION OF PARASITAEMIA LEVEL

The level of parasitaemia was determined by using a haemocytometer (Neubauer Improved) (Subekti et al. 2013). Using wet tail-blood film, it was estimated every 2 days for 4 weeks. When parasitaemia levels were elevated, a drop of blood (10 µL) was placed on a microscope slide and mixed with SDS 1% (1:1). As soon as possible, mixed blood was collected using micropipette and diluted in Phosphate Saline Glucose (PSG) 1:100 (if the level of parasitaemia was $10^5$-$10^6$ parasites/mL) or 1:1000 (if the parasitaemia level was greater than $10^7$) and homogenised. Furthermore, the number of parasites was estimated using this formula (Subekti et al. 2013):

$$\text{Parasite count/mL} = A \times B \times 10^4$$

where A is the number of parasites count in leucocyte chamber; and B is the dilution of blood before counting.

### STATISTICAL ANALYSIS

Statistical Package for the Social Sciences (SPSS) 23 software was used to conduct the statistical analyses. The mean and standard error of the mean were used to present the data (SE). For survival analysis, the log rank test was used.

## Results and Discussion

### PREPATENT PERIOD, SURVIVAL TIME AND VIRULENCE

The isolates of *T. evansi* used in this study had variable prepatent periods ranging from 2.0 to 5.3 days' post-infection (dpi) (Table 2). The shortest prepatent period (2 dpi) occurred most frequently in mice (59.4%), followed by 2.4 to 2.6 dpi, 3.3 and 4.0 to 5.3 dpi which occurred in 21.9%, 3.1% and 15.63%, respectively. Our findings indicated that the virulence level of 32 *T. evansi* isolates from Indonesia was not always correlated with prepatent period. According to Bengaly et al. (2002), *T. evansi* isolates with a high virulence tend to possess a short prepatent period. Subekti et al. (2013) corroborated this statement by observing that the majority of mice infected with a high virulent isolate of *T. evansi* expired within a short period of time (less than 1 week). Theoretically, the causative agent of disease is positively correlated with the prepatent period based on genetic type, and mice succumbed rapidly following detection of parasites in peripheral blood. Nonetheless, numerous *T. evansi* isolates from this study produced inconsistent results. Although some of the mice infected with low-virulence isolates, such as Pdg 382 and Pdg 384, had a short prepatent period (2 days post-infection), they had a long survival time of 23.7 and 27.3 dpi, respectively. In mice infected with high-virulence *T. evansi* isolates from Sumbawa, distinct phenomena occurred (Sbw 340 and Sbw 363 isolates). Despite their short survival time, the mice exhibited prolonged prepatent periods of 4 dpi and 3.3 dpi, respectively. The findings of this

study corroborated those of De Menezes et al. (2004), who reported prepatent periods in susceptible (C3H) and resistant (Swiss Webster) mice following infection with *T. evansi*. Both mouse strains had nearly identical prepatent periods (approximately 3.5 dpi), but their survival times were significantly different. Swiss Webster mice lived approximately twice as long as C3H mice, indicating that there is no correlation between prepatent periods and parasite virulence in different host resistant strains.

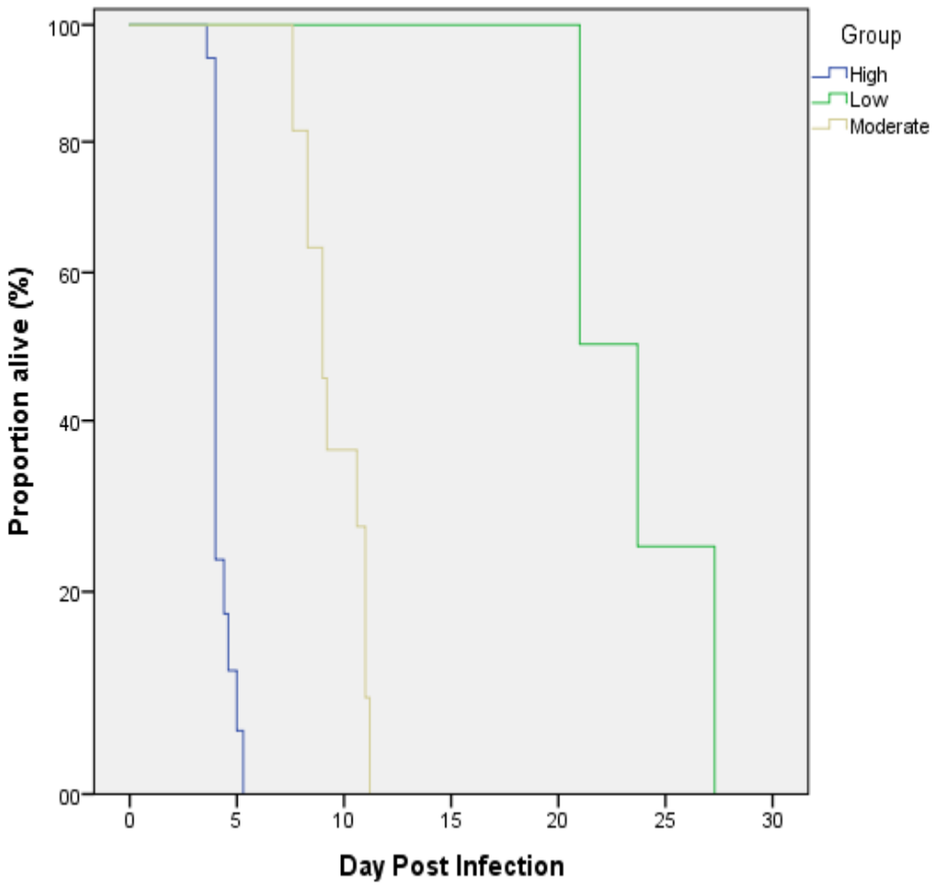
TABLE 2. Prepatent period, survival time and virulence of Indonesian *T. evansi* in mice

| No. | Name isolate | Prepatent period [a] | Survival time[a] | Virulence[b] |
|---|---|---|---|---|
| 1 | Bang 87 | 2.0 ± 0.00 | 4.0 ± 0.00 | High |
| 2 | Bang 85 | 2.0 ± 0.00 | 4.0 ± 0.00 | High |
| 3 | Pml 287 | 2.6 ± 0.35 | 21.0 ± 0.47 | Low |
| 4 | Pml 291 | 2.6 ± 0.35 | 21.0 ± 0.62 | Low |
| 5 | Sbw 340 | 4.0 ± 0.00 | 5.0 ± 0.33 | High |
| 6 | Sbw 341 | 2.0 ± 0.00 | 4.0 ± 0.00 | High |
| 7 | Sbw 363 | 3.3 ± 0.35 | 4.0 ± 0.00 | High |
| 8 | Sbw 364 | 2.0 ± 0.00 | 4.0 ± 0.00 | High |
| 9 | Sbw 366 | 2.0 ± 0.00 | 4.0 ± 0.00 | High |
| 10 | Smi 68 | 2.0 ± 0.00 | 4.0 ± 0.00 | High |
| 11 | Smi 369 | 2.0 ± 0.00 | 4.4 ± 0.24 | High |
| 12 | Bwi 218 | 2.0 ± 0.00 | 4.0 ± 0.00 | High |
| 13 | Bwi 222 | 2.0 ± 0.00 | 5.3 ± 0.25 | High |
| 14 | Bwi 228 | 2.6 ± 0.36 | 4.6 ± 0.25 | High |
| 15 | Ash129 | 2.0 ± 0.00 | 8.3 ±0.35 | Moderate |
| 16 | Ash 133 | 2.0 ± 0.00 | 7.6 ± 0.50 | Moderate |
| 17 | Smb 370 | 2.0 ± 0.00 | 8.6 ± 0.70 | Moderate |
| 18 | Smb 371 | 5.3 ± 0.35 | 9.0 ± 0.62 | Moderate |
| 19 | Smb 372 | 2.0 ± 0.00 | 4.0 ± 0.00 | High |
| 20 | Smb 373 | 2.6 ± 0.35 | 9.0 ± 0.66 | Moderate |
| 21 | Smb 374 | 5.3 ± 0.35 | 9.2 ± 0.62 | Moderate |
| 22 | Smb 375 | 4.0 ± 0.00 | 11.0 ± 0.00 | Moderate |
| 23 | Lbk 376 | 2.4 ± 0.40 | 3.6 ± 0.26 | High |
| 24 | Bgr377 | 2.0 ± 0.00 | 4.0 ± 0.00 | High |
| 25 | Pdg 378 | 2.6 ± 0.35 | 10.6 ± 0.60 | Moderate |
| 26 | Pdg 379 | 4.0 ± 0.00 | 11.0 ± 0.30 | Moderate |
| 27 | Pdg 380 | 2.6 ± 0.35 | 8.3 ± 0.58 | Moderate |
| 28 | Pdg 381 | 2.0 ± 0.00 | 11.2 ± 0.63 | Moderate |
| 29 | Pdg 382 | 2.0 ± 0.00 | 23.7 ± 0.67 | Low |
| 30 | Pdg 384 | 2.0 ± 0.00 | 27.3 ± 0.50 | Low |
| 31 | Pdg 386 | 2.0 ± 0.00 | 4.0 ± 0.00 | High |
| 32 | Pdg 388 | 2.0 ± 0.00 | 4.0 ± 0.00 | High |

Other studies confirmed the findings of this current study, demonstrating that *T. evansi* isolates from Indonesia had comparable prepatent periods to those from Brazil, but were shorter than those from the Philippines when inoculated into susceptible mouse strains. *T. evansi* isolates from Luzon and Mindanao had a prepatent period of 5.3 to 10.9 days, respectively, in susceptible BALB/c mice, whereas *T. evansi* from Brazil had shorter prepatent period of 2.4 to 3.7 dpi in the same mouse strain (De Menezes et al. 2004).

The reason why Indonesian isolates of *T. evansi* with short prepatent periods had such a long survival time is likely due to the low virulence of *T. evansi* isolates that are well adapted to various hosts in the field. As a result, the parasite multiplies rapidly in mice but takes a long time to kill the host. Another observation was that *T. evansi* isolates with a high level of virulence had lengthy prepatent periods and a short survival time (Sbw363 and Sbw340). This can be attributed to the strong immune response of the host, which is capable of inhibiting parasite multiplication during the early stages of infection. However, over time, the parasite was able to evade the host immune response. The parasites multiplied rapidly in order to reach a state of parasitaemia, which resulted in the death of mice in a short period of time (5 dpi).

According to Holzmuller et al. (2008) and Magez et al. (2004), if one strain grows more rapidly than another, it may result in a more severe disease, but there is no absolute correlation between parasite number and pathology severity.




[a]Survival rate (Av.) ± standard error (SE), [b]Strains were considered to be of high and low virulence when the Av. + SE was less than 5.4 days and when the Av. − SE was more than 17 days, respectively. Strains that did not fit into either of the preceding two categories were considered to be of moderate virulence

FIGURE 1. The survival curves of mice infected with either high, moderate or low virulent *T. evansi* isolates. The Kaplan-Maier method was used to construct curves of survival. The prepatent period and the mean survival time are shown in Table 2. The log rank test was used to determine the differences among groups infected with low, moderate and high virulent isolates ($p<0.01$)

### DISTRIBUTION OF THE VIRULENCE OF *T. evansi* IN MICE BASED ON MEDIAN SURVIVAL TIME

The median survival time of *T. evansi*-infected mice was variable (3.6 to 27.3 dpi) (Table 2). The shortest survival time was detected in mice infected with the Lbk 376 isolate (3.6 ± 0.26 dpi), while the longest survival time was observed in mice infected with the Pdg 384 isolate (27.3 ± 0.5 dpi).

According to the median survival time (Table 2, Figure 1), the virulence level of *T. evansi* can be classified as high, moderate or low. *T. evansi* isolates killed mice in less than 1 week in 53.13% of cases (3.6 to 5.3 dpi). This group was classified as having a high level of virulence. Around 34.38% of *T. evansi* isolates were classified as having a moderate virulence and killing mice within 1 to 2 weeks. The remaining isolates (12.5%) were classified as having low virulence, capable of killing mice after more than 2 weeks (Table 3).

The result also showed that the prepatent period did not always correlate with the median survival time and level of virulence. Short prepatent periods have not always been associated with increased virulence, and vice versa. Mekata et al. (2013) classified virulent *T. evansi* isolates from the Philippines into three categories namely high, moderate and low virulences. The group of high virulence was characterised as having a median survival time plus standard error (SE) (less than day 12.86 PI), whereas the group of low virulence had a median survival time greater than day 14.56 PI. The remaining isolates were classified as having a moderate level of virulence (between 12.86 and 14.56 PI). This classification was also used to determine the virulence of *T. congolense* (Masumu et al. 2006). In this present study, the survival time of infected mice also demonstrated the virulence level of *T. evansi* (3.6 to 27.3 PI). *T. evansi* with a high virulence level killed mice in 3.6 to 5.3 PI, with a moderate virulence level in 7.6 to 11.2 PI, and with a low virulence level in 21 to 27.3 PI.

Although Mekata et al. (2013) reported that the median survival time of *T. evansi* isolates from the Philippines ranged from 12.86 to 14.56 dpi, but it cannot be concluded that *T. evansi* isolates from Indonesia are more pathogenic than those from the Philippines. According to Verdillo et al. (2012), *T. evansi* isolates from the Philippines collected from Luzon, Visayas and Mindanao island exhibited varying degrees of virulence. Visayas isolates were able to kill mice on 3 dpi and 4 dpi by 70% and 30%, respectively, whereas Luzon isolates killed mice on 5 dpi (90%) and 4 dpi (10%). Additionally, the Mindanao isolates could kill mice at 4 dpi and 6 dpi for 10% and 20%, respectively.

These reports corroborated our findings. The isolates collected from different islands exhibit varying degrees of virulence. On 5 days post-infection, the isolates from Sumbawa Island and Bangkalan (high virulence) could kill 100% of mice. In comparison to Verdillo et al. (2012), this study also showed variation of virulence in the same village. While isolate 372 from the village Wajelo (Sumba) killed 100% of mice on day 5 PI, another isolate from the same village (Sbw 375 isolate) killed only 67% and 33% of mice on days 6 and 14, respectively (Table 2). The discrepancy between the current study and the study by Verdillo et al. (2012) was attributed to the number of tested isolates. They used a small number of isolates from the Philippines, which limited their ability to fully describe the variation in virulence among *T. evansi* stocks from that country.

### PATTERNS OF PARASITAEMIA AND MORTALITY IN MICE

This study established that parasitaemia peaked at $10^7$ to $10^8$ parasites/mL of peripheral blood (Figure 2). Groups of isolates classified as having a high virulence category (17 isolates) rapidly reached a peak of parasitaemia on 4th dpi ($10^8$ *T. evansi*/mL of blood), followed by complete death of mice (100%) on 5th to 6th dpi. For the moderate (11 isolates) and low (4 isolates) virulence categories, parasitaemia peaked later, on 8th dpi. The mice retained a peak of parasitaemia for several days and died between the 8th and 14th dpi (moderate virulence), or after 16 dpi (low virulence). Low-virulence parasites exhibited fluctuating parasitaemia patterns. Interestingly, the majority of the mice in the moderate virulence group presented parasitaemia patterns that alternated between high and low virulence.

Based on the level of parasitaemia results, *T. evansi* in mice can be divided into several patterns (Figure 2; Table 3). The isolates with a high level of virulence exhibited only one pattern of parasitaemia, i.e., pattern 1 (Figure 2(a)). The isolates with a moderate level of virulence exhibited four parasitaemia patterns, namely patterns 2-1, 2-2, 2-3 and 2-4 (Figure 2(b), 2(c), 2(d) and 2(e)). Additionally, the isolates with low virulence exhibited three parasitaemia patterns, namely 3-1, 3-2 and 3-3 (Figure 2(f), 2(g) and 2(h)).

The parasitaemia patterns can be used to assess the virulence of *T. evansi* in mice. De Menezes et al. (2004) identified three distinct parasitaemia patterns in susceptible (BALB/c and C3H) and resistant (C57BL/6 and Swiss-Webster) mouse strains. The first type of mice developed undulating parasitaemia and died 28 days after infection. For the second type, mice were able to

maintain parasitaemia at or below $10^6$ parasites/mL of blood for 10 days, but then parasitaemia increased to $10^8$ to $10^9$ parasites/mL of blood, and mice died after 26 days. Lastly, the third type, mice with parasitaemia levels of $10^8$ to $10^9$ parasites/mL of blood died 12 days after infection. Interestingly, the present study also demonstrated three distinct parasitaemia patterns in mice based on the virulence of *T. evansi* (Table 3).

TABLE 3. Parasitaemia pattern of Indonesian *T. evansi* isolates based on level of virulence

| Parasitaemia pattern | Virulence | Isolate | Total sample | Percentage |
|---|---|---|---|---|
| | High | Bang 87, Bang 85, Sbw 340, Sbw 341, Sbw 363, Sbw 364, Sbw 366, Smi 68, Smi 369, Bwi 218, Bwi 222, Bwi 228 | 17/32 | 53.13% |
| 2-1 | Moderate | Pdg 379 | 1/32 | 3.13% |
| 2-2 | Moderate | Asah 129, Asah 133, Sbt 370, Sbt 371, Sbt 374, Pdg 380 | 6/32 | 18.75% |
| 2-3 | Moderate | Smb 373, Smb 375, Pdg 378 | 3/32 | 9.38% |
| 2-4 | Moderate | Pdg 381 | 1/32 | 3.13% |
| 3-1 | Low | Pml 287, Pml 291 | 2/32 | 6.25% |
| 3-2 | Low | Pdg 382 | 1/32 | 3.13% |
| 3-3 | Low | Pdg 384 | 1/32 | 3.13% |

Pattern 1 was detected in 17 *T. evansi* isolates. This pattern was characterised by parasitaemia peak at 4 dpi, followed by the death of all mice at 5 to 6 dpi, which was observed in isolates with high virulence (Table 3). This pattern was not found in the study by De Menezes (2004) for either sensitive or resistant mice strain. Pattern 3 was defined by the presence of undulating parasitaemia which was observed in isolates with low virulence (Figure 2(f), 2(g) & 2(h)). Compared to the research of De Menezes et al. (2004), the three sub-patterns were detected in the resistant mouse strains (C57BL/6 and Swiss-Webster) that were infected with highly virulent *T. evansi* from Brazil. Both of these findings established that the parasitaemia pattern of *T. evansi* was dependent on parasite virulence and mouse strain. Resistant mouse strains infected with highly virulent *T. evansi* had the same parasitaemia pattern as susceptible mouse strains infected with low virulent *T. evansi*.

Patterns 3-1, 3-2 and 3-3 were characterised by the presence of undulating parasitaemia. These patterns were detected in four *T. evansi* isolates. The pattern of 3-1 was characterised by undulating parasitaemia throughout the life time of the mice (Figure 2(f)). Pattern 3-2 exhibited double-undulating parasitaemia (Figure 2(g)), whereas pattern 3-3 exhibited no undulating parasitaemia (Figure 2(h)). Waves of parasitaemia associated with low-virulent isolates may be related to the parasite immune control by the host. When relapse occurs, the parasite evades the host immune response, allowing the parasites to remain in the host for an extended period of time. This assertion was consistent with the observations of Magez et al. (2020) that trypanosomes have evolved a quorum sensing system capable of regulating proliferation in response to parasite density and also of sensing excessive infection-associated inflammatory tissue damage in order to ensure trypanosome survival in the host for an extended period of time. This regulation is frequently observed in isolates with low virulence.

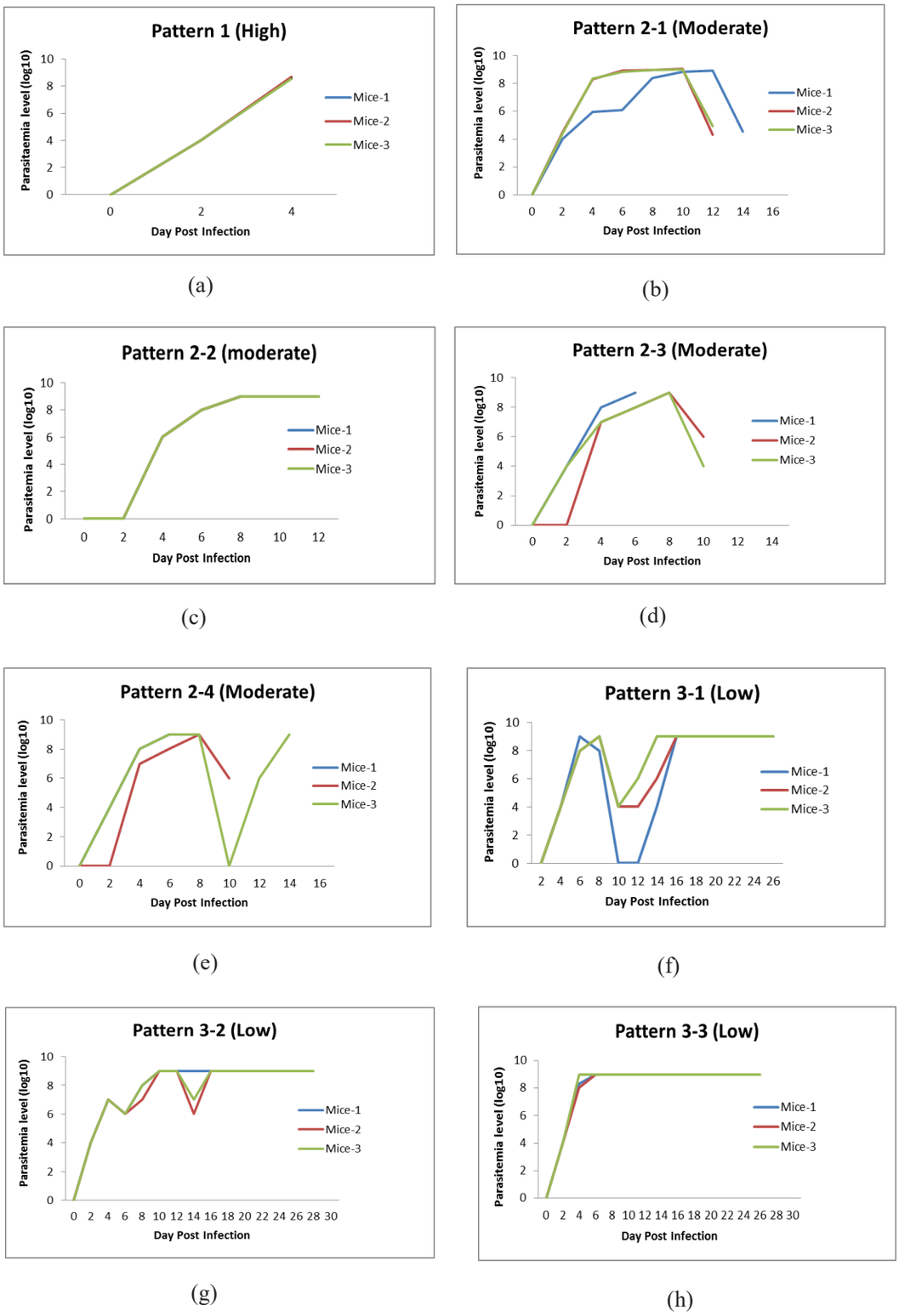



FIGURE 2. Parasitemia patterns in mice infected by $10^4$ *T. evansi*/mouse. The patterns were a) Pattern 1 (high virulence), b) Pattern 2-1 (moderate virulence), c) Pattern 2-2 (moderate virulence), d) Pattern 2-3 (moderate virulence), e) Pattern 2-4 (moderate virulence), f) Pattern 3-1 (low virulence), g) Pattern 3-2 (low virulence), and h) Pattern 2-3 (low virulence)

Pattern 2 was defined as a combination of patterns 1, 2 and 3 that occurred in isolates of *T. evansi* with moderate virulence. This could indicate the presence of two or more distinct virulent strains (high and low) infecting the mice in the same group. This pattern was observed among 11 *T. evansi* isolates. The peak of parasitaemia was maintained in this pattern until mice died at 14 dpi or later (Figure 2(b), 2(c) and 2(d)). The patterns 2-1, 2-2, 2-3 and 2-4 were characterised by the presence of a single peak of parasitaemia at 6 to 8 dpi, then followed by a decline in the parasitaemia at 12-14 dpi, and the death of mice. Pattern 2-4 has double peak parasitemia and the death of mice 12-14 dpi. The 2-2, 2-3 and 2-4 patterns were combination between patterns 1 and 3-3 (Figure 2(c)), patterns 1 and 2-1 (Figure 2(d)), and patterns 1 and 2-1 (Figure 2(e)). According to Sawitri and Wardhana (2019), *T. evansi* with moderate virulence can be a mixed infection (containing both high and low virulent strains) or a single strain. For mixed infections, if the proportion of parasites with low virulence is greater than 50%, the population will be classified as isolates with low pathogenicity. Conversely, if the proportion of parasites with high virulence is greater than 50%, the population will be classified as parasites with moderate pathogenicity. De Menezes et al. (2004) observed pattern 2-1 in susceptible mice (BALB/c and C3H/He) infected with highly virulent *T. evansi*. These patterns may reflect three capacities of the host immune system namely an inability of the immune system to cope with the first wave of parasitaemia (one peak and death), an ability of the immune system to cope with the first peak parasitaemia but not with the second peak (2 peaks and death), and an ability of the immune system to cope with the first two peaks parasitaemia and death (3 peaks and death).

In contrast, immune control is not possible in mice infected with highly virulent isolates. As a result, the parasite does not relapse and the host dies shortly after the parasitaemia peaks. According to Black, Seed and Murphy (2001), susceptible hosts produce antibodies specific to VSG resulting in a repeating wave of parasitaemia. However, because the development of VSG-specific antibodies takes several days and the multiplication of trypanosomes takes approximately 7 hours, the inability of the host immune response to divert the changes of the trypanosome VSG results to an uncontrolled parasitic multiplication and immune system depression. Eyob and Matios (2013) stated that trypanosomes suppress both humoral antibody and T cell-mediated immune responses, causing the host immune system to fail to respond to consistent parasitic load or secondary infection, resulting to the development of immunopathological lesions.

The parasitaemia pattern of *T. evansi* in mice demonstrated unequivocally that there were multiple strains of *T. evansi* present in the same or different isolates derived from the same or different water buffaloes in the same or different geographical regions. The host response shapes the course of the disease, and genetic variation within the host can influence disease severity, particularly in the mouse model (Morrison 2011).

Based on the observations in infected livestock in the field, circulating *T. evansi* isolates from the outbreak and endemic areas in Indonesia are dominated by isolates with moderate to low virulence (67.85%) and mild clinical symptoms, such as emaciation, weakness and poor appetite have been observed among infected water buffaloes. This condition could occur because majority of the highly virulent isolates may die concurrently with the hosts. Another hypothesis is that natural selection occurs in high virulence *T. evansi* that loses to low or moderate virulence *T. evansi*. As a result, the proportion of isolates with low to moderate virulence is high within an animal population or geographic area. Observations conducted in areas where trypanosomosis is endemic and susceptible cattle breeds are the primary vector hosts suggest that the reservoir of trypanosomes may favour the persistence of trypanosome strains (den Bossche 2001). It is critical to keep in mind that low and moderate quantities of T. evansi isolates may favor possible reservoirs. Masumu et al. (2006) supported this statement by stating that because the dominant strains of *T. evansi* in the field are moderate and low virulent isolates, it will significantly contribute to the sustainability of circulating parasites in cattle population because of their role as a reservoir. According to Mekata et al. (2013), highly virulent isolates in mice induce severe high clinical symptoms when transferred to cattle. Contrary to popular belief, this does not occur with low virulent isolates. Similar results were also reported by Bengaly et al. (2002), where *T. congolense* exhibiting high virulence in mice also showed high virulence in cattle. Thus, the expression of virulence of *T. evansi* in mice may be representative of these isolate's characteristics in cattle. According to Sawitri dan Wardhana (2019), buffalo infected with *T. evansi* have clinical symptoms such as fever, anemia, depression and weakness, and cause a high rate of mortality in outbreak areas in Indonesia. However, in endemic areas, the majority of the cattle and buffalo infected with *T. evansi* with moderate or low virulence exhibit no clinical symptoms and die infrequently or not at all.

## Conclusions

*T. evansi* isolates from Indonesia exhibited a range of virulence classified into three categories namely high, moderate and low virulence. Each category has a distinct prepatent period, a distinct parasitaemia pattern, and a distinct mortality time in experimental mice. Often, there is no absolute correlation between prepatent periods, parasite counts, and mortality. Since *T. evansi* isolates with low and moderate virulence are the most prevalent types circulating in the field, it is necessary to be aware of their role as reservoir by infecting the host with no clinical signs.

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*Corresponding author; email: dyah.haryuningtyas@gmail.com