Genetic Variability of ESAG 6/7 Gene Isolat *Trypanosoma evansi*

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ABSTRAK

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Trypanosoma evansi (*T. evansi*) sebagai agen penyakit Sura merupakan salah satu penyakit parasitik penting untuk diperhatikan karena dapat menimbulkan kerugian ekonomi yang sangat besar di Indonesia. Parasit ini memerlukan zat besi untuk fase propagasi yang diperoleh dari pejamu melalui reseptor transferrin (Tf) yang dikode oleh *Expression Site Associated Genees* (ESAGs). ESAG6/7 dilaporkan mengkode reseptor transferrin pada afinitas yang berbeda pada pejamu yang berbeda.Adanya perbedaan patogeneitas *T. evansi* diduga menyebabkan variabilitas pada gene ESAG 6/7. Penelitian ini bertujuan untuk melihat variabilitas gene ESAG6 *T. evansi* dengan virulensi yang berbeda pada mencit. Penelitian ini dilakukan dengan 2 tahap yaitu uji patogeneitas *T. evansi* pada mencit dan analisis sekuen gene ESAG6/7. Uji patogeneitas dilakukan dengan melihat median lama hidup mencit setelah masing-masing kelompok diinfeksi dengan 25 *T. evansi* asal kerbau dari berbagai kondisi geografi. Hasil penelitian uji patogeneitas menunjukkan adanya perbedaan virulensi pada 25 isolat T.evansi pada mencit. Hasil analisis sekuensing gene ESAG6/7 dari 25 isolat *T. evansi* asal Indonesia cenderung homogene pada daerah pengikatan transferin (Tf) tetapi ditemukan adanya variabilitas pada hiper variable site. Perubahan tersebut mampu memisahkan isolat *T. evansi* virulensi tinggi dan rendah. Hasil analisis pohon filogenetika terbentuk 11 clade dari 25 isolat *T. evansi*. Isolat dengan virulensi tinggi termasuk dalam clade 7 dan 10. Isolat dengan virulensi rendah masuk dalam clade 5 dan 11. Isolat dengan virulensi moderat terbagi dalam dua *clade* tersebut.

Kata Kunci: *T. evansi*, Gen ESAG6/7, Variabilitas, Virulensi

ABSTRACT

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Trypanosoma evansi as an agent of Surra is one of the crucial parasitic diseases that cause great economic losses in Indonesia. These parasites need iron for growth and propagation phase which is obtained by receptor-mediated uptake of host transferin. The transferrin receptors are encoded by Expression Site Associated Genees (ESAGs). ESAG6/7 encodes transferrin receptors which reported have different affinities of a different host. The distinction of *T. evansi* pathogenicity is supposed to cause variability in the ESAG6/7 gene. This research was aimed to investigate the variability of genes ESAG6/7 *T. evansi* with different virulence in mice. This research was conducted in two steps: bioassay pathogeneicity in mice and analysis of ESAG6/7 gene sequences. The median survival time of mice was investigated after each group of mice infected by 25 *T. evansi* isolates from buffaloes where its geographically differ. The test results showed a difference of pathogenic virulence on 25 *T. evansi* isolates in mice. Sequence analysis of the ESAG6/7 gene from 25 *T. evansi* isolates origin from Indonesia tended to be homogeneous on the transferrin binding site but there was variability in the hypervariable site. These changes are able to separate high and low virulence of the *T. evansi* isolates. Phylogenetic tree analysis was formed 11 clades of 25 *T. evansi*. High virulence *T. evansi* was included in clades 7 and 10, while low virulence *T. evansi* was included in clade 5 and 11 and the moderate virulence was divided into those four clades.

Key Words: *T. evansi*, ESAG6/7 Gene, Variability, Virulence

INTRODUCTION

Trypanosomiasis caused by *T. evansi* has widely spread South America, Africa and Asia (Sarkhel et al. 2017). This blood protozoon is the cause of Surra disease which is one of the crucial parasitic diseases on the animal in Indonesia. Almost in all area in Indonesia is an endemic area of Surra. The disease has caused huge economic losses on the late case in Sumba during 2010-2012 (direct communication with East Sumba Livestock Services 2012) and in Pandeglang, Banten during 2013-2014 (direct communication with Indonesian Research Center for Veterinary Sciences, 2014).

Various biochemical and molecular typing on *trypanosomiasis* have been widely developed to describe the correlation between species and subspecies (Amer et al. 2011). *Trypanosoma evansi* consists of a

large number of morphologically identical populations that significantly different on various biological characters such as host range, virulence, pathogenicity and drug sensitivity (Sarkhel et al. 2017). Reid (2002) stated that there are different clinical manifestation and geography differences of isolate that supposed to show high genetic diversity between isolates, especially related to pleiotropism phenomenon (single gene that causing various phenotypic). This was supported by study result of Masiga et al. (2006) who found a genetic diversity of *T. evansi* from Kenya according to the amplified fragment length polymorphism/AFLP test (Masiga et al. 2006). A genetic variable of *T. evansi* was also reported based on the *expression-site-associated* gene *e* 6/7 (ESAG6/7) as a marker (Mekata et al. 2009; Villareal et al. 2013). Trypanosome is able to avoid the immune attack of the host by changing the expression of Variant Surface Glycoprotein (VSG) genes. Variant Surface Glycoprotein expressed was located in the end part of the telomere, the polycistronic transcription unit called VSG expression site (VSG-ESs) (Hutchinson et al. 2016). The polycistronic VSG ESs consisted of a number of Expression Site Associated Genes (ESAGs) which one of it had been characterized as ESAG6/7 involved in the nutrient acquisition (Schell et al. 1991).

Trypanosome takes iron in the blood circulation through high-affinity receptor mediated by an iron-bound transferrin endocytosis called holo-Tf. This receptor was encoded by homolog ESAG6 and 7 genes (Sarkhel et al. 2017). These genes were reported able to detect *T. evansi* genetic diversity in South America (Mekata et al. 2009). As much as 20 variants have been identified (Witola et al. 2005). Variable Surface Glycoprotein Expression Site (ES) forms complex nucleotide pattern as long as 40-60kb. The upstream part of the VSG gene is repeated unit as long as 70bp which is suspected to play a role in the gene conversion. At the part of ES promotor and upstream part of the VSG, there is minimal six different open reading frame (ORF) called expression-site-associated genee (ESAG). In the blood circulation, *T. evansi* used Tf receptor to take the whole iron (Fe) from the host blood for growth and multiplication **(**Kabiri & Steverding 2001; Sarkhel et al. 2017**).**

Transferrin is a protein from a beta globulin group that binds and distributes iron in the blood serum. Transferrin receptor is encoded by two homolog expression-site-associated genees (ESAG6 and ESAG7) located in VSG area. Transferrin receptor was heterodimer receptor linked by Glycosylphosphatidylinositol (GPI) (Mehlert et al. 2012). As much as 20 ESAG 6/7 variants were equivalent with the number of VSG expression site but there was only one that active sometimes (Isobe et al. 2003; Witola et al. 2005). That two ESAG only showed the different of a sequence about 1-10% (Witola et al. 2005; Mekata et al. 2009). Despite that small difference, it was reported to be able to cause affinity difference of transferrin host (Bitter et al. 1998; Steverding et al. 1995). Bitter et al. (1998) reported that natural variability of ESAG6/7 might affect the ability of trypanosome to take Tf molecule from various species of Mammalia as host. Isobe et al. (2003) and Witola et al. (2005) reported that on *Trypanosoma equiperdum*, where this parasite only infected horse, has known as ESAG6 gene, which was less diverse compared to *T. brucei and T. evansi* infecting wide range host (Isobe et al. 2003). This indicated that the diversity of ESAG6/7 gene had an important role in the ability of parasite to adapt the different hosts (Mekata et al. 2009).

Nevertheless, a hypothesis of a chance of *T. brucei* to express different Tf receptor related to the Tf difference obtained on various mammalian species (Gerrits et al. 2002). Recent results indicated that iron storage caused an increase of 3-10 times from Tf area along with redistribution of the receptor from flagellar pocket to entire parasite surface (Mehlert et al. 2012). On the cronical infected animal, the Tf level of host decreased then trypanosome developed an ability to grow on low iron concentration by increasing the level of expression area of Tf so that increase iron infusion (van Luenen et al. 2005). Therefore, activity or inactivity of transcription from VSG Es gene was a mechanism of very active regulation causing the parasite quickly responses the environment change. Therefore, the ESAG6/7 sequence had high polymorphism on site which related to Tf binding part contributed on iron requirement fulfilling and its adaptation on the different host (Gerrits et al. 2002). This gene was also suspected to be able to describe genetic diversity of *T. evansi* that allegedly related with pathogeneicity difference (Witola et al. 2005)**.**

The result of pathogeneity level study on mice showed that nine Indonesian *T. evansi* isolated from different geographic had different pathogeneicity level on mice (Wardhana et al. 2011). This pathogenetic level difference is allegedly to cause genetic diversity of ESAG 6/7 *T. evansi* gene of Indonesian isolates that have not been studied so far. This study was conducted to observe the variability of ESAG6/7 gene on various *T. evansi* isolates from the same host that had different virulence on mice. The existence of variability on this gene is expected able to be used as marker to distinguish the virulence of local *T. evansi* isolate. This marker then can be used to help determine the spread of *T. evansi* isolate in Indonesia according its virulence.

MATERIALS AND METHODS

Ethics committee

This research has been approved by the Ethics Committee of the Faculty of Medicine, University of Indonesia with the number of Ethics 24 / H2.F1 / ETIK / 2013.

Trypanosoma evansi isolate

As much as 25 Trypanosome sp isolates were used in this study were the Culture Collection of the Indonesian Research Center for Veterinary Sciences (BCC) for 1985-2008 and osilates from outbreak site during 2012-2014 (Table 2). Those isolates were stored as stabilate in the form of cryopreservation using glycerol as the cryoprotectant.

Table 1. Samples of *T. evansi* isolates used in this study

Isolate code	BCC code	Isolate source (Sub District, District, Province)	Year of isolation	Type of animal	Information
Bang 85		Bangkalan, Bangkalan East Java	1988	Buffalo	BCC
Bang 87	P0176	Bangkalan, Bangkalan, East Java	1988	Buffalo	BCC
Pml 287	P232	Pemalang, Pemalang, Central Java	1996	Buffalo	BCC
Pml 291	P233	Taman, Pemalang, Central Java	1997	Buffalo	BCC
Sbw 340	P202	Lape Lopok, Sumbawa Besar, West Nusa Tenggara	1998	Buffalo	BCC
Sbw 341	P203	Lape Lopok, Sumbawa Besar, West Nusa Tenggara	1998	Buffalo	BCC
Sbw 366	P029	Lape Lopok, Sumbawa Besar, West Nusa Tenggara	1999	Buffalo	BCC
Smi 68	P169	Surade,Sukabumi, West Java	1985	Buffalo	BCC
Smi 369	P125	Surade, Sukabumi, West Java	2008	Buffalo	BCC
Smb 370	-	Wajelo, Sumba Timur, East Nusa Tenggara	2012	Buffalo	Outbreak, Dept of Paracitology
Smb 371	-	Wajelo, Sumba Timur, East Nusa Tenggara	2012	Buffalo	Outbreak, Dept of Paracitology
Smb 372	-	Wajelo, Sumba Timur, East Nusa Tenggara	2012	Buffalo	Outbreak, Dept of Paracitology
Smb 373	-	Wajelo, East Sumba, NTT	2012	Buffalo	Outbreak, Dept of Paracitology
Smb 374	-	Wajelo, East Sumba, East Nusa Tenggara	2012	Buffalo	Outbreak, Dept of Paracitology
Smb 375	-	Wajelo, East Sumba, East Nusa Tenggara	2012	Buffalo	Outbreak, Dept of Paracitology
Lbk 376	-	Lebak, Banten	2013	Buffalo	Outbreak, Dept of Paracitology
Munt377	-	Muntilan, Central Java	2013	Buffalo	Endemic, Dept of Paracitology
Pdg 378	-	Cisata, Pandeglang, Banten	2013	Buffalo	Outbreak, Dept of Paracitology
Pdg 379	-	Cisata, Pandeglang, Banten	2013	Buffalo	Outbreak, Dept of Paracitology
Pdg 380	-	Cisata, Pandeglang, Banten	2013	Buffalo	Outbreak, Dept of Paracitology
Pdg 381	-	Cisata, Pandeglang, Banten	2013	Buffalo	Outbreak, Dept of Paracitology
Pdg 382	-	Carita, Pandeglang, Banten	2014	Buffalo	Outbreak, Dept of Paracitology
Pdg 384	-	Carita, Pandeglang, Banten	2014	Buffalo	Outbreak, Dept of Paracitology
Pdg 386	-	Cisata,Pandeglang, Banten	2014	Buffalo	Outbreak, Dept of Paracitology
Pdg 388	-	Cisata, Pandeglang, Banten	2014	Buffalo	Outbreak, Dept of Paracitology

Experimental animals

As much as 80 male mice of DDY of 10-12 weeks old with body weigh around 25-30 g were obtained from the National Agency of Drug and Food Control, Jl. Percetakan Negara, Jakarta. Those mice were fed with commercial pellets (Indofeed) in the morning and evening as much as 5-10 g per head/day. The drinking water was offered *ad libitum*. Twenty mice were used for *T. evansi* multiplication and those 60 mice were used for biological test.

***T. evansi* multiplication on mice**

Isolates stored with cryopreservation in the liquid nitrogen was thawed first then diluted using Phospat Buffer Saline Glucose (PBSG) up to 0.2 mL and injected (0.1 mL/mice) intraperitoneally (IP) to multiply the parasite infection material. Parasitemia level of experimental animal was evaluated every two days. When the parasitemia level reached the peak (about 10^7-10^8 *trypanosoma*/mL blood), the experimental animal were sacrificed and the blood was collected. The blood containing *T. evansi* was used as the source of infection to be injected to the experimental animal by 10^4 parasites per mice intraperitoneally (IP) and partially used for DNA isolation.

***T. evansi* counting**

Parasite counting was conducted to obtain parasite concentration by 1x 10^4 parasite/ml blood to be tested its virulence on mice and for observation of parasitemia level every two days. For the parasitemia tested in every two days, the blood samples were collected from vena tail: the end of tail was cleaned using alcohol 70% then wounded. The dripping blood was collected by micropypet (± 10 µL), then mixed with SDS 1% (1 : 1) until homogeny. The dilution was then diluted immediately using PBSG with 1 : 100 or 1 : 1000 ratio according to its patacytemia level and examined using hemocytometer (Naubeuer Improved) (Subekti et al. 2013). The number of parasite was counted in leucocyte counting room using the following formula:

The number of parasite/mL = A x B X 10^4

A = The number of *trypanosoma* counted on the leucocyte room
B = dilution factor

Pathogenicity test of *T. evansi* on mice

Determination of pathogenicity level was to identify *T. evansi* isolates which were categorized as high, moderate and low virulence. As much as 25 *T. evansi* isolates were infected to mice by 10^4 parasite/0.3 mL dose. Each isolate was infected into 3 male mice. The observation of the experimental animal death was performed twice a day along with the feeding time.

Criteria of virulence level were in accordance with Wardhana et al. (2011):

- High virulence: when the tested isolates were able to kill mice within less than one week.
- Moderate virulence: when the tested isolates were able to kill the mice in 8 days-2 weeks
- Low virulence: when the tested isolates were able to kill the mice in more than 2 weeks.

DNA extraction

DNA extraction of *Trypanosoma sp* on the mice blood was performed using Genomic DNA Mini Kit (Geneaid, Taiwan) following the procedure offered in the kit. The extracted DNA of each isolate was filled in the 1.5 mL tube, be labeled and stored at -20°C for further analysis.

PCR optimization of ESAG 6/7 *T. evansi* gene

PCR amplification was performed using Primer ESAG 7 Forward (F): 5'-CATTCCAGCAGGAGTTGGAGG-3' and ESAG 6 Reverse (R): 5'-TTGTTCACTCACTC TCTCTTTGACAG-3' and ESAG 6 Reverse (R): 5'-TTGTTCACTCACTC TCTCTTTGACAG-3' on ABi GeneAmp Thermal Cycler 9700 machine. Each reaction has total volume by 25 µl using KAPA2G^{TM} Fast PCR kit (KAPA BIOSYSTEMS, USA). PCR condition in this study was pre-denaturation (95°C, 3 minutes, 1 cycle); denaturation (95°C, 10 seconds, 35 cycles); primer attachment (58°C, 15 seconds, 35 cycles); DNA fragment extension (72°C, 15 seconds, 35 cycles) and the last DNA fragment extension (72°C, 10 minutes, 1 cycle). Amplification results were visualized on 1.5% agarose gel using SYBR® Safe gel staining (Invitrogene^{TM}) with electrophoresis technique (100 volt 20 minutes). The visualization and vaccinated DNA bands analysis were performed in the GelDoc Transluminator (Cleaver).

Sequencing and phylogenic tree analysis of ESAG 6/7 *T. evansi* gene

Sequencing process was performed using ABI Prism 3.1.1 sequencing machine. Sequence of gene was edited using DNAbaser version 4 (BioSoft 2013) software. Translation of DNA sequence into protein was performed using translation program (http: //tw expasy.org/tools/dna.html). Sequence alignment and

phylogenic tree were constructed using MEGA program version 6 (Tamura et al. 2013). Calculation of genetic distance value used neighbor joining genetic distance method with 2 parameters Kimura substitution model (K2P) with 2000 replications.

Table 2. Sequence reference of *Trypanosoma* on ESAG6/7 site from gene bank for amino acid analysis

Access number	Species/Isolate	Host	Location	Reference
AB 551909	*T. evansi*	Camel	Egypt	Amer et al. 2011
AB551912	*T.evansi*	Camel	Egypt	Amer et al. 2011
AB551914	*T. evansi*	Camel	Egypt	Amer et al. 2011
AB551917	*T.evansi*	Camel	Egypt	Amer et al. 2011
AF068704	*T. brucei rhodesiense*	-	-	Pedram and Donelson, unpublished
AF068705	*T. brucei rhodesiense*	-	-	Pedram and Donelson, unpublished
EU726388	*T. brucei rhodesiense*	-	-	(Young et al. 2008)
EU726354	*T. brucei gambiese*	-	-	(Young et al. 2008)
EU726385	*T. equiperdum*	-	-	(Young et al. 2008)
EU726387	*T.equiperdum*	-	-	(Young et al. 2008)
EU726388	*T.equiperdum*	-	-	(Young et al. 2008)
EU726389	*T.equiperdum*	-	-	(Young et al. 2008)
EU726392	*T.equiperdum*	-	-	(Young et al. 2008)
EU726431	*T.brucei brucei*	-	-	(Young et al. 2008)
XM 840855	*T. brucei brucei*	-	-	-
KR 858296	*T. evansi*	-	-	-
KR 858299	*T.evansi*	-	-	-

RESULTS AND DISCUSSION

Pathogenicity test on mice

Pathogenicity is generally determined by infectivity and virulence. Infectivity is defined as the ability of parasite to multiply and stay alive on the host, while virulence is the capacity of parasite to damage and cause a disease. Study of T. evansi from Brazil showed the virulence heterogeneity of isolate and laboratory animal pathogenicity (Queiroz et al. 2000; de Menezes et al. 2004). Herrera et al. (2001) reported that artificial infection on the domestic and wild animal showed virulence difference and pathology change.

This study results showed that life time of mice in each group that infected by 25 *T. evansi* isolates from Indonesia was around 4-28 days. Those results could be divided into three categories: high, moderate and low. High virulence was when the mice death less than one week, moderate virulence was when the mice death between 7-14 days and the low virulence was when the mice death more than two weeks (Table 4). Those categories was consistent with the research result of Wardhana et al. (2011).

According to virulence of T. evansi isolate and the number of mice death on the 30 days observation, it may be categorized into three groups: group I (high virulence), group II (low virulence) and group III (moderate virulence) with life time median by 0+ 0.25 day: 21.0+0.47-28.4+0.68 days and 7.6+0.71- 9.0 + 0.64 days (Table 4). This result was similar with the T. evansi isolates from Philippine reported by Mekata et al. (2013) who reported that the virulence of T. evansi isolate from Philippine was divided into three categories: high virulence when the life time median + standard error (SE) was less than 12.86 days after infection (hpi) and low virulence when the life time median + standard error (SE) was more than 14.56 hpi. Isolates that did not be categorized into those two categories was considered as moderate virulence.

This research result also showed that isolates from the same location might have same or different virulence. As an example, Sumba isolate from same location had two virulence categories as high virulence (Smb372) and moderate virulence (Smb370, 371, 373, 374, 375). This was similar with T. evansi isolate from Philippine. Verdillo et al. (2012) stated that T. evansi isolate from Philippine collected from Luzon, Visayas

and Mindanao Islands, showed different virulence. Branda˜ et al. (2002) and O'Garra (1998) stated that the pathogenicity of Surra depended on strain virulence, infection route and individual sensitivity of host.

Table 3. Sequence reference of *Trypanosoma* on ESAG6/7 site from gene bank for clade determination

Access number	Species/Isolate	Location	Reference
AB179565	*T. evansi*	Thailand	Witola et al. 2005
AB179576	*T. evansi*	Thailand	Witola et al. 2005
AB179578	*T. evansi*	Thailand	Witola et al. 2005
AB179594	*T. evansi*	Thailand	Witola et al. 2005
AB179599	*T. evansi*	Thailand	Witola et al. 2005
AB179604	*T. evansi*	Thailand	Witola et al. 2005
AB179607	*T. evansi*	Thailand	Witola et al. 2005
AB179617	*T. evansi*	Thailand	Witola et al. 2005
AB179613	*T. evansi*	Thailand	Witola et al. 2005
AB496616	*T. evansi*	Peru	Mekata et al. 2009
AB496621	*T. evansi*	Peru	Mekata et al. 2009
AB496622	*T. evansi*	Peru	Mekata et al. 2009
AB496623	*T. evansi*	Peru	Mekata et al. 2009
AB496626	*T. evansi*	Peru	Mekata et al. 2009
AB496627	*T. evansi*	Peru	Mekata et al. 2009
AB496629	*T. evansi*	Philippine	Mekata et al. 2009
AB496630	*T. evansi*	Philippine	Mekata et al. 2009
AB496631	*T. evansi*	Philippine	Mekata et al. 2009
AB496633	*T. evansi*	Philippine	Mekata et al. 2009
AB496638	*T. evansi*	Philippine	Mekata et al. 2009
AF068702	*T. brucei rhodesiense*	-	Alarcon, Pedram, and Donelson 1999
AF068703	*T.brucei rhodesiense*	-	Alarcon, Pedram, and Donelson 1999
AJ007021	*T. brucei brucei*	-	Ansorge et al. 1999
AJ007023	*T. brucei brucei*	-	Ansorge et al. 1999
AJ007026	*T. brucei brucei*	-	Ansorge et al. 1999
AY152684	*T. equiperdum*	-	Isobe et al. 2003
AY152688	*T.equiperdum*	-	Isobe et al. 2003

Sequencing Analysis of ESAG6/7 *T. evansi* gene

To determine the genetic variability of ESAG6/7 of Indonesian *T. evansi* isolate, as much as 25 *T. evansi* isolates with different virulence were analyzed along with *T. evansi*, *T. brucei* and *T. equiperdum* from GeneBank in this study. Multiple Sequence Alignment and phylogeny tree analysis of amino acid sequence was confirmed that there were 4 variants of the total 25 *T. evansi* isolates (Figure 2 and 3). Only a small part of the amino acid sequence showed any variability. However, Witola et al. (2005) and Mekata et al. (2009) reported that ESAG6/7 gene only showed sequence difference about 1-10%. Despite its small difference, but its reported to cause affinity difference of different transferrin host (Bitter et al. 1998). In contrast to the *T. evansi* isolates from Thailand, Indonesian *T. evansi* isolates tend to be homogeneous on the transferring binding site located on box II and III (Figure 3) (Salmon et al. 1997). Isobe et al. (2003) stated that

Table 4. Median of long life, death and virulence of *T. evansi* on mice

Isolate	Median of long life ± SE day)	Dominant mice death	Virulence
Bang85	4±0.00	< 7 hpi	High
Bang87	4±0.00	< 7 hpi	High
Pml287	21±0.47	>15 hpi	Low
Pml291	21±0.62	>15 hpi	Low
Smi68	4±0.00	< 7 hpi	High
Smi369	4±0.00	< 7 hpi	High
Sbw 340	5±0.00	< 7 hpi	High
Sbw 341	4±0.00	< 7 hpi	High
Sbw 366	4±0.00	< 7 hpi	High
Smb370	7.6±0.71	7-14 hpi	Moderate
Smb371	9±0.66	7-14 hpi	Moderate
Smb372	5±0.00	7-14 hpi	High
Smb373	9±0.62	7-14 hpi	Moderate
Smb374	9±0.64	7-14 hpi	Moderate
Smb375	11±0.00	7-14 hpi	Moderate
Lbk376	4±0.25	< 7 hpi	High
Mun377	4±0.00	< 7 hpi	High
Pdg378	10.6±0.62	7-14 hpi	Moderate
Pdg379	11±0.33	7-14 hpi	Moderate
Pdg380	8.3±0.58	7-14 hpi	Moderate
Pdg381	11±0.63	7-14 hpi	Moderate
Pdg382	24.4±0.51	>15 hpi	Low
Pdg384	28.4±0.68	>15 hpi	Low
Pdg386	4+0.00	>15 hpi	High
Pdg388	4+0.00	>15hpi	High

ESAG6/7 variant of *T. equiperdum* that had been cloned and characterized, was less varied on the transferrin binding site compared to the *T. brucei*. The *T. equiperdum* only infect horse; so that the genetic diversity disappeared along with the absence of selection (Moran 2002). It was allegedly that ESAG6/7 gene became more homogeny on the area that was not influenced by positive selection including the Tf binding site (Witola et al. 2005). Low viability value in this study could be explained due to the *T. evansi* used in this study, which was from the same host, buffalo. Nevertheless, it was found amino acid variability located on the hyper variable site (HV). Variation of amino acid residual on the HV site and/or on the Tf binding site might help the parasite to avoid immune response. The HV site had played a role in anticipating antigenic surface variation and not involved in the Tf binding site (Salmon et al. 1997).

Even though the 25 *T. evansi* isolates in this study were from the same host, but it showed different virulence on mice from various location with different geographic. Therefore, the amino acid change on that position might affect antigenic characteristic of the residue located on the receptor surface and would be exposed to the outside environment

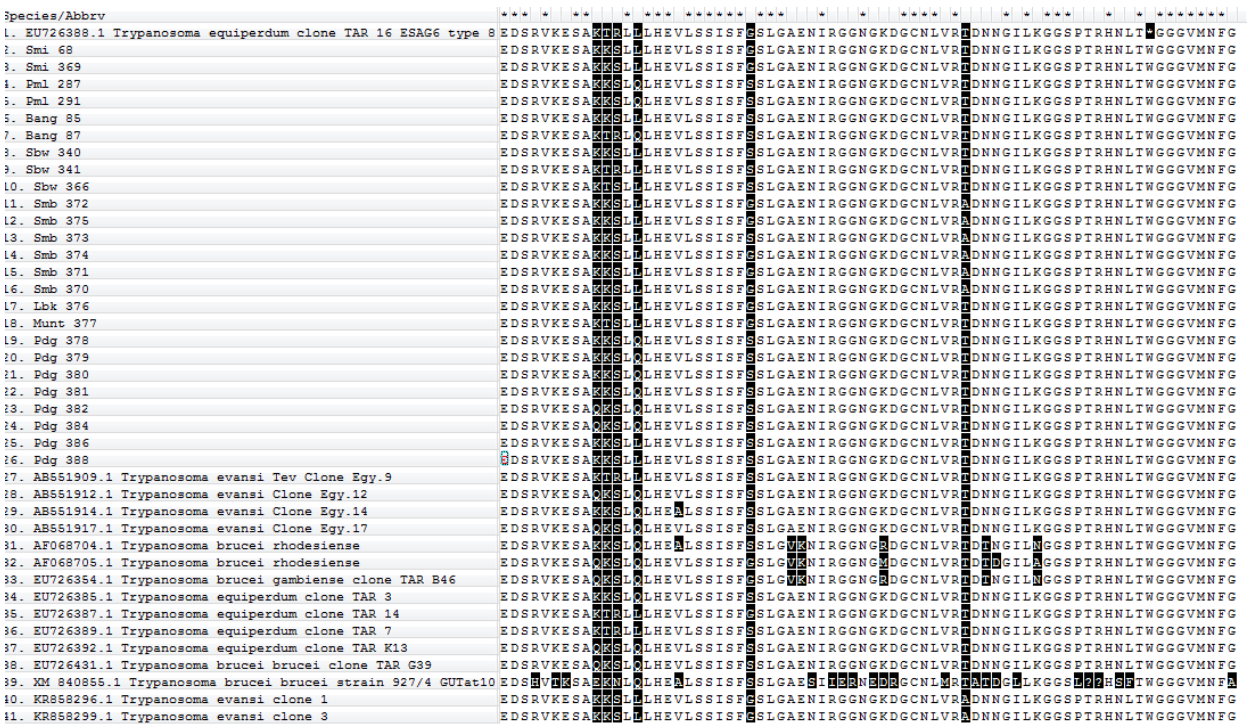

Figure 2. Sequence alignment of amino acid of ESAG6/7 on 25 Indonesian *T. evansi* isolates compared to *T. evansi, T. brucei* and *T.equiperdum* sequences form GeneBank. Two protein locations of ESAG6 which is a part of transferrin improvement site (I and II , Salmon et al. (1997) that showed by the box.

Salmon et al. (1997) stated that the sequence variation on transferrin binding site on the ESAG6/7 gene clearly seen on *T. equiperdum* and *T. brucei* did not play a role on improvement of the transferrin on antigenic variation on the receptor surface due to a mutation in this site but did not significantly influence the Tf improvement. Tf I receptor, which was look very much like its structure with VSG. Amino acid sequence on the very strategic position on the Tf receptor indicated that its residue was on the loop which would be exposed to the outside environment (Salmon et al. 1997). Even it was a site with high variability and located on the end molecule, but it was not yet certain whether it would be exposed and antigenic. Antigenic variation influenced was the one that was located on the receptor surface (Isobe et al. 2003). Even the *T. equiperdum* was closely related to the *T. brucei*, due to no selection pressure for telemeric maintenance of VSG ES. The number of copy of genetic variability and the selection power of this telemeric sequence would also decrease (Isobe et al. 2003).

The control of antigenic variation was influenced by two main things: the virulence and avoiding strategy of immune response, and expression of mono-allele gene (Lyons et al. 2013). Glover et al. (2013) described that trypanosome used about one-third of nucleus gene and all sub telomere for activating and inactivating the VSG gene and the sequence related to the VSG. Variation of antigenic needs a single VSG transcription by polymerase RNA I (Pol-I) and periodically change of gene expression, especially through DNA recombination by duplicating the translocation into new VSG on the new active site. Furthermore telomere location, epigenic control and mono-allele transcription by Pol-I on the extra nucleolar site is important for the VSG expression by telomere, chromatin structure. Nucleus organization played a role on the contribution to the control and expression activity change of mono-allele VSG.

Analysis of phylogenic tree of *T. evansi* according to amino acid sequence of ESAG 6/7 gene from GeneBank

The phylogeny tree of amino acid sequence of ESAG 6/7 gene of 25 Indonesian *T. evansi* isolates was compared to some reference isolates from Gene Bank produced three groups (Figure 3). Bitter et al. (1998) reported that there was 20 pairs of ESAG 6 gene (20 gene variants) equivalent to VSG expression site number, but it was only one pair that active sometimes. The difference of amino acid between isolates was only 1-10% (Bitter et al. 1998; Steverding et al. 1995). This was affected by incompatibility of the number of copies

of genes due to the effects of homogenization through gene and telomere conversion mechanism (Robinson et al. 1999). Glover et al. (2013) described that VSG ES was the polycistron key unit involved in antigenic variation on trypanosome. Function and expression of this unit highly influences the virulence. In trypanosome maturation from mRNA transcription occurs through trans-slicing process, there were addition of 39 capped sequences and polyadenilasi ESAG6 / 7.

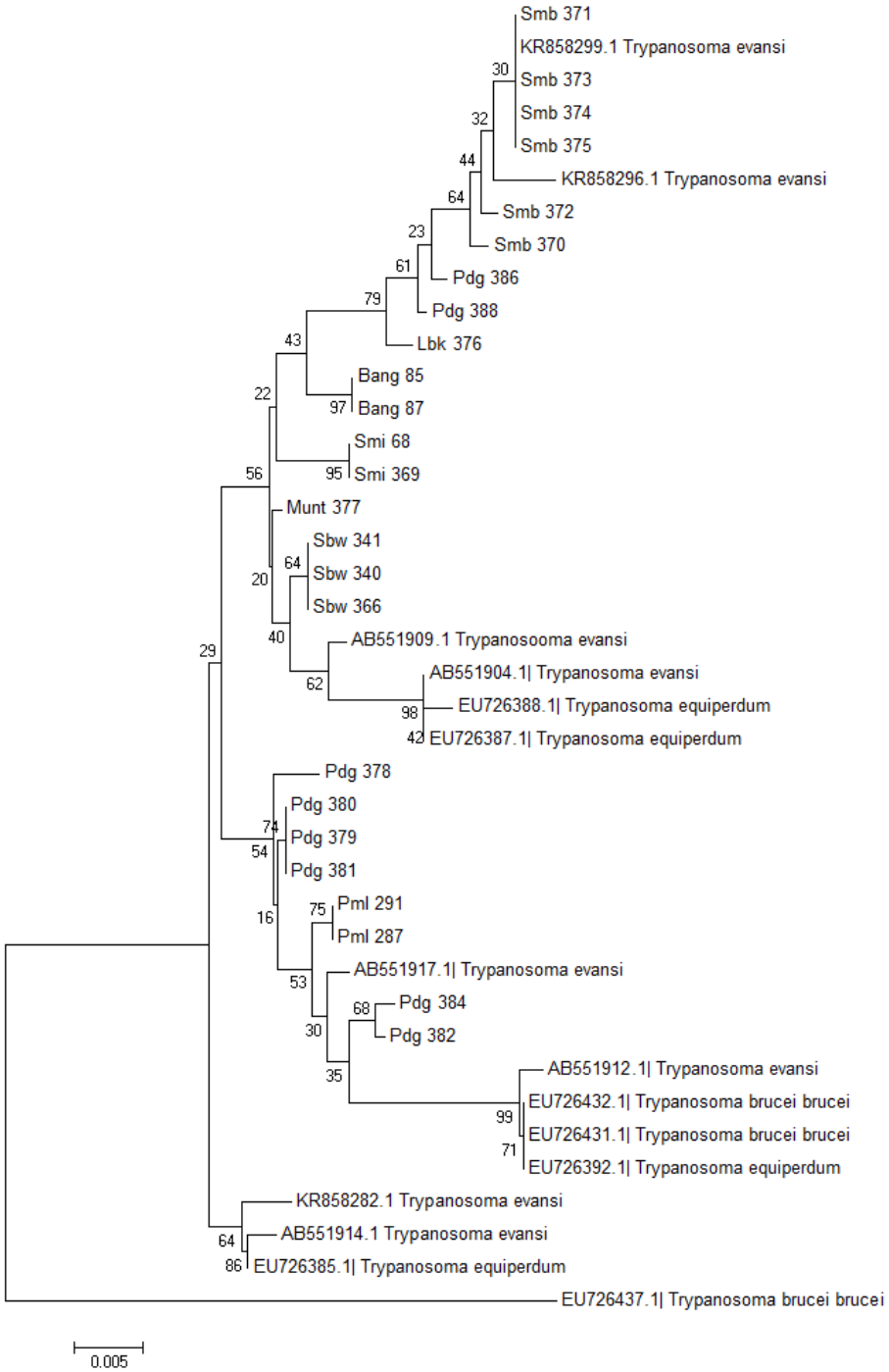

Figure 3. Neighbour joining phylogenic tree based on amino acid sequence of ESAG6/7 *T.evansi, T.brucei T. equiperdum* genes from GeneBank (access number is behind the isolates). Genetic distance between sequences is showed by number.

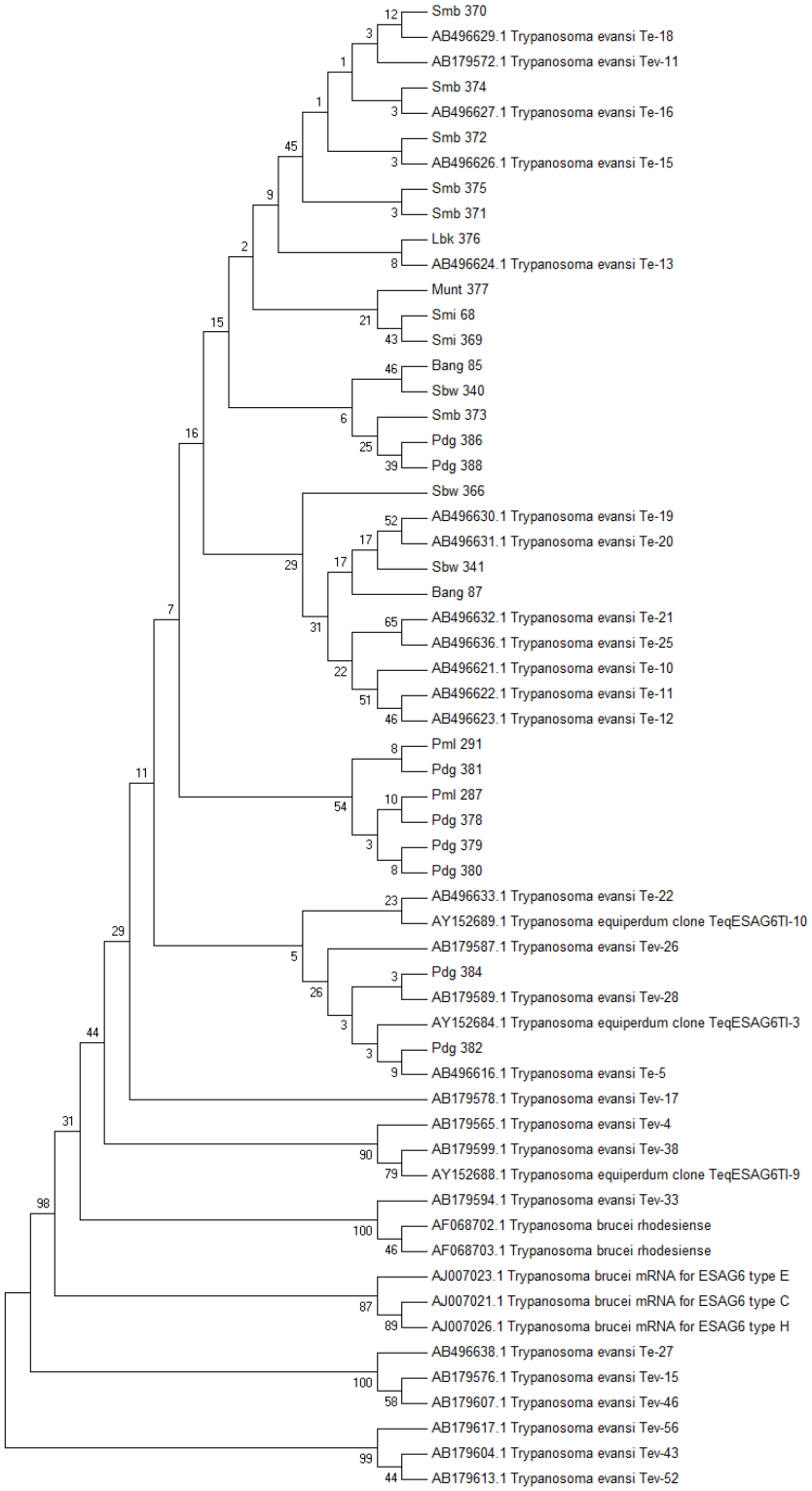

Figure 4. *Neighbour joining* of phylogeneic tree based on amino acid sequence of variant ESAG 6 *T.evansi (Tev), T.brucei brucei, T.brucei gambiense, T.brucei rhodesiense, T. equiperdum* genees (Tbb, Tbg, Tbr, Teq respectively) from Gene*eBank* (access number is behind the isolates). Geneetic distance between squences is showed by number.

Even though, isolates showed high homology but had a separate position in the phylogenetic tree. That pattern showed heterogeneity on the sequence although very small.

Analysis of phylogenic tree of *T. evansi* according to amino acid sequence of ESAG 6/7 gene for clade determination

Phylogenic tree construction was built according to mode used by Mekata et al. (2009). This construction was built according to 10 categories of ESAG 6/7 gene clade (Figure 3). As much as 25 Indonesian *T. evansi* isolates from various geographic areas with different virulence divided into 4 clades (*Clade* 5, 7, 10, and 11). The most Indonesian *T. evansi* isolates were from ESAG 6 and 7 genes of *T. evansi* from Southeast Asia (Witola et al. 2005) and South America (Mekata et al. 2009) (Figure 2). As much as 13 Indonesian *T. evansi* isolates were clade 7 and two isolates were clade 5. However, there were 6 isolates not included in 10 clades categorized in the previous study (Mekata et al. 2009; Barghash et al. 2016). Those isolates were Pdg 378, Pdg 379, Pdg 380, Pdg 381, Pdg 287 and Pdg 291. Those six isolates created new clade (clade 11). This new clade 11 was found on the Indonesian *T. evansi* isolates. Research result of Witola et al. (2005) on the *T. evansi* phylogenic tree analysis from Thailand showed 7 clades, while the research result of Mekata et al. (2009); Barghash et al. (2016) and Sarkhel et al. (2017) on *T. evansi* each from United State, Egypt and India showed 10 clades.

ESAG6gene as pathogenicity marker of *T. evansi*

Analysis result of phylogenic tree showed that isolates categorized to low virulence, was apart from the high virulence (Figure 3). However, moderate virulence isolates were spread in to low and high virulence. Dominant low virulence isolates would be merged with the low virulence isolates. While, dominant moderate virulence similar with high virulence isolates were merged with the high virulence isolates. Therefore, ESAG 6/7 gene was potential to be used as virulence marker. As well as the analysis result of phylogenic tree on the clade determination showed that high virulence isolates were categorized as different clade with low virulence isolates (Figure 4). High virulence was categorized into clade 7 and 10, while low virulence isolates was in clade 5 and 11. Bitter et al. (1998) described that natural variability of ESAG 6/7 was able to influence the trypanosome ability to take transferrin molecule from some species that was different from mammalian host. The variation of sequence, especially on HV site was related to its role on antigenic variation on the receptor surface to avoid immune response of host (Borst 1991). This was suspected that the sequence was playing a role in chronic diseases, related to parasite pathogenicity and its ability to widely infect host (Pays et al. 2006).

Some studies were expected that the variation on receptor development site was one of factors that contributed on parasite pathogenicity difference and its ability to widely infect the host (Pays et al. 2006). According to the amino acid diversity, among those, 25 *T. evansi* isolates were monophyletic group. It might be caused due to the only certain species that were infected by *T. evansi* in that site. It was suspected that the *T. evansi* on the site was stuck in the buffalo for a long time, so that it genetic diversity on the ASEG 6/7 gene was loss and became homogeny and there was no polymorphism on the transferrin development site (Mekata et al. 2009). Bitter et al. (1998) stated that sequence diversity could be also caused by the number of this gene copy. Variability of ESAG 6/7 allowed trypanosome to express the receptor with different affinity on the different host (Salmon et al. 1997) indicating the ability of parasite to adapt on the host (Amer et al. 2011).

CONCLUSION

As much as 25 Indonesian *T. evansi* isolates were divided into 4 clades of EASG 6/7 clades. Low virulence isolates were categorized as clade 7 and 10. Several low virulence isolates was in clade 11 (the new clade). Genetic variability occurred in the hipervariable site (HVR) but not in the Transferin binding site of ESAG 6/7 gene. According to this ESAG 6/7 gene, the Indonesian *T. evansi* was able to be distinguished into high and low virulence isolates. While the moderate virulence was spread on that two groups.

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