

Molecular Identification of ABC2 Transporter Gene Encode Protein Ngawi *Trypanosoma evansi* Isolate that suspected resistance to Isometamidium Chloride

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Abstract. This study aims to determine the profile of the ABC2 encoding transporter on *Trypanosoma evansi* (*T. evansi*) Ngawi isolates, Indonesia, exposed with Isometamidium Chloride (ISM). This study used blood samples of mice containing *Trypanosoma evansi* that had been exposed with ISM 0.05 mg/kg BW, ISM 0.1 mg/kg BW and ISM 0.3 mg/kg BW for 4 weeks, and control group. Blood samples were extracted and amplified using primers. ABC2 F 5 'GCTTGTCGACCATCTTGCA 3' and ABC2 R 5 'AGGTCCACTCCCATGCTACA 3' that produced 350 basepairs (bp). The sequencing results were then analyzed using BLAST and MEGA 7.0. There was 1 deference nucleotide (107) derived from multiple alignments, while in amino acids there was no difference in all samples. *Trypanosoma evansi* which was exposed with ISM does not have many differences in nucleotide or amino acid and only one type of mutation. The ABC2 Transporters of four groups of *T. evansi* have high similarity to ABC Transporters of *T. brucei gambiense*, *T. brucei brucei*, and *T. brucei brucei* (Tbabc2). Therefore, further research on the ABC2 Transporter gene is needed.

Key Words: ABC2 Transporter Gene, *Trypanosoma evansi*, *Isometamidium Chloride*, resistance.

1 Introduction

Surra is a disease caused by blood protozoa, namely *Trypanosoma evansi*. This disease can infect animals in tropical and semitropical regions (Nurcahyo 2017). *Trypanosoma* transmission can occur mechanically through insect bites (Desquesnes et al. 2013). *Trypanosoma evansi* can infect wild and domestic animals. Wild animals which can become hosts are elephants, whereas hosts from domestic animal groups can be camels, cows, buffaloes, horses, and small ruminants (Davila et al. 2000; Desquesnes 2004; Herrera et al. 2005). Cases of trypanosomiasis have been reported to infect dogs, carnivorous animals (Hosseini et al. 2007). *Trypanosoma evansi* infection is very pathogenic to cows, horses, camels, and Asian buffaloes (Desquesnes et al. 2013). The disease can cause large economic losses for the livestock industry in endemic countries. The surra outbreak caused economic losses of 22.4 million US dollars per year in Indonesia (Partoutomo et al. 2000). Furthermore, to reduce the impact of surra's disease, the government recommends trypanocidal drugs.

Trypanocidal is a group of drugs that is used as curative (medication) and preventive actions in surra cases.

The drugs included in this group are isometamidium chloride, cymelarsan (melarsormine dihydrochloride), suramin, quinapyramine, and diminazene aceturate (Giordani et al. 2016; Steverding 2010). Two types of trypanocidal are commonly used in Indonesia, isometamidium chloride (ISM) and diminazene aceturate (Subekti 2014). The unappropriated dose of these drugs might cause decreased sensitivity or resistance to the drugs (Sinyangwe et al. 2004). As reported by Subekti et al. (2015), several trypanocidals are not effective for Indonesia's Sura Cases. Cases of trypanosoma resistance to the drugs have been reported such as in West Africa's *Trypanosoma vivax* and *Trypanosoma congolense* (Mongube et al. 2012; Sow et al. 2012; Vitouley et al. 2012). Other cases of trypanocidal resistance in Ethiopia have been reported in several studies (Moti et al. 2012; Dagnachew et al. 2015; Mekonnen et al. 2017). Melaku and Birasa (2013) reported trypanocidal resistance cases in 17 African countries which increased to 21 cases in 2018 (Assefa and Shibesi 2018). The case of resistance to isometamidium does have many pathways, but several studies have shown that there is a close relationship between ISM resistance and the encoding gene of ATP-Binding Cassete (ABC) Transporter protein (Beis, 2015; El-Awady et al., 2017; Nuryady et al., 2021; Tihon et al., 2017).

ATP-Binding Cassete (ABC) is one of the largest protein transporters for families and can be found in

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prokaryotic and eukaryotic organisms. ABC transporters which are owned by eukaryotes only functioned as efflux proteins, whereas influx and efflux protein functions can be found in prokaryotic organisms (Higgins 2001; Breier et al. 2013; Videira et al. 2014). ABC transporters such as P-glycoprotein are one of the factors that influence cases of drug resistance related to efflux (Borst and Ouellette 1995). Several studies have shown cases of resistance that are thought to be related to the ABC transporter gene. ABC transporters owned by protozoa, function as influx and efflux proteins (Balakrishnan et al. 2004). Research Delespau et al. (2005), showed that there was a mutation in the transmembrane protein in the mitochondria which was suspected as ABC transporter so that it affected the resistance of the trypanocidal drug. This research was carried out using isolates of *Trypanosoma congolense*, while screening of the gene encoding ABC2 Transporter family in *Trypanosoma evansi* isolates had never been done and the gene is not yet available in Genebank data, herefore this study will screen and see the sequential profiles of genes encoding ABC2 Transporter in *Trypanosoma evansi* isolates exposed with isometamidium chloride stress.

2 Material And Method

2.1 Ethical Clearance

The use of mice animal model has been fulfilled the ethic eligibility that has been approved by the Ethical Clearance commission, Faculty of Veterinary Medicine (FKH), Gadjah Mada University (UGM), Yogyakarta, Indonesia with number: 0024 / EC-FKH / Int. / 2018.

2.2 *Trypanosoma evansi* sample

The samples used in this study were 88 blood samples taken from cows in Ngawi Regency, East Java, Indonesia. The sample examination was carried out at the Wates Veterinary Center using blood smear preparations and microhematocrit. One of the samples was *Trypanosoma evansi* positive and was suspected to be resistant to Isometamidium Chloride by random selection then inoculated to mice.

2.3 Propagation of *T. evansi* in mice

The mice used in this study came from the Integrated Research and Testing Laboratory (LPPT), Unit VI, UGM, Yogyakarta, with the following criteria: Balb-C, male, 8 weeks old, and body weight + 25 grams. *Trypanosoma evansi* inoculation was carried out after the mice were acclimatized for 1 week. At the beginning of the experiment, mice were infected with 10^5 *T. evansi* / mice intraperitoneally. One day after the mice were inoculated, blood samples were collected through coccygeal venous to determine the level of parasitemia under a microscope. A high level of parasitemia or 4+ is indicated by the presence of more than 20 *T. evansi* / field of view (10^8 *Trypanosoma* / ml) under a microscope with 200x magnification. The mouse is used

as a source of propagation to 20 other mice. *T. evansi* propagation was carried out intraperitoneally using a physiological saline solution as a diluent as much as 0.3 ml and mixed with the blood of mice that had been inoculated with *T. evansi*. Parasitemia was observed one day after inoculation.

2.4 Administration of ISM

Mice that had been inoculated with *T. evansi* were divided into 4 groups, namely groups A, B, C, and D. Observation of parasitemia was done two days after inoculation. Isometamidium chloride (ISM) exposure was applied if each group showed parasitemia 10^7 *Trypanosoma* / ml with the following conditions: group A (control) was given a placebo, group B, C, and D with ISM doses 0.05, 0.1, 0.3 mg/kg body weight. The blood of mice was collected at the end of the study (day 25), the blood collection was carried out through retro-orbitals after the mice were euthanized. Blood is inserted into the EDTA tube and stored at -20 °C for DNA extraction (Sawitri and Wardhana 2017).

2.5 DNA Extraction

One blood sample of mice from each group was taken as a representative group. The DNA from the sample was extracted using QIAamp® DNA Blood Mini Kit with the following procedure, blood containing 5-10 µl EDTA was dissolved in PBS to a volume of 220 µl in 1.5 ml of sterile tubes, then 20 µl of proteinase K was added. Then 200 µl of Buffer AL was added and vortexed for 10 seconds, and then incubated in a water bath at 56 °C for 10 minutes. A 96% ethanol solution was added as much as 200 µl, then the tube was vortexed for 10 seconds. The vortex process results in solution and precipitate in the tube, the solution is then pipetted and transferred into a 2 ml tube in DNase Mini Spin Column. The tube was centrifuged at 8000 rpm for 1 minute, then the collection tube contained the filtrate was removed. The Spin Column is moved into a new collection tube, then 500 µl Buffer AW1 was added. In the next step, the Spin Column was centrifuged at 8000 rpm for 1 minute, then the collection tube contained the filtrate was removed. The Spin Column was then transferred to the new collection tube and 500 µl AW2 buffer was added to the tube. The tube was centrifuged at 14,000 rpm for 3 minutes and the collection tube containing the filtrate was removed. The Spin Column was transferred to a new collection tube and 200 AL buffer was added, then incubated at room temperature for 1 minute. In the next stage, the tube was centrifuged at 8000 rpm for 1 minute. The solution produced after centrifugation was pipetted and transferred into the microtube and stored at -20 °C.

2.6 DNA Amplification

The results of DNA isolation were then used as a template for the amplification process using the PCR method. The amplification target was the ABC2 Transporter protein-coding gene in the *T. evansi* isolate

(~ 350 bp). Primers were prepared using online primers3 based on the Tbac2 gene sequencing in Genbank Accession No. U89027.1. The primers used in Tbac2 amplification were 5 'GCT TGT CCG ACC ATC TTG CA 3' as forward primer and 5 'AGG TCC ACT CCC ATG CTA CA 3' as reverse primer. DNA amplification with PCR was done by mixing 5 µl samples, 12.5 µl Master mix, 1 µl (10 pmol ABC2 F primers), 1 µl (10 pmol ABC2 R primers), and 5.5 µl ddH2O to obtain a total volume of 25 µl in the PCR tube. The PCR conditions used for amplification are as follows: initial denaturation at 94 °C for 4 minutes, followed by denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 30 seconds performed 35 cycles and then terminated with termination at 72 °C for 5 minutes. The PCR products were visualized in 1,5 % gel agarose with 100 voltages in 30 minutes.

2.7 Data Analysis

PCR results were sequenced at PT. Genetics Science, Jakarta. Sequences were then analyzed with BLAST and MEGA software version 7.0.

3 Results and Discussion

Table 1. The genetic distance of ABC2 Transporter gene of *Trypanosoma evansi*.

	A	B	C	D	E	F	G
A							
B	0,000						
C	0,000	0,000					
D	0,000	0,000	0,000				
E	0,003	0,003	0,003	0,003			
F	0,003	0,003	0,003	0,003	0,000		
G	0,003	0,003	0,003	0,003	0,000	0,000	

Informations: A: *T. evansi* A, B: *T. evansi* B, C: *T. evansi* C, D: *T. evansi* D, E: *T. b. gambiense* ABC Transporter, F: *T. b. brucei* ABC Transporter, G: *T. b. brucei* (Tbac2)

The amplification of ABC2 F and ABC2 R primers produced 350 basepair (bp) PCR products (Figure 1). The sequencing results of samples were then analyzed using BLAST and showed 99.36% homology to the ABC Transporter putative gene from *Trypanosoma brucei gambiense* (XM 011779815.1), and *Trypanosoma brucei brucei* (XM 817911.1) based on GenBank. The result of multiple alignments between the ABC2 nucleotide protein transporter from GenBank and samples obtained 318 basepairs. Based on the results of the alignment between the results of sample sequences and the comparative ABC Transporter protein, there was only one different nucleotide (Figure 2). The difference of nucleotide is shown in base 107 (Thymine / T replaced by Cytosine / C).

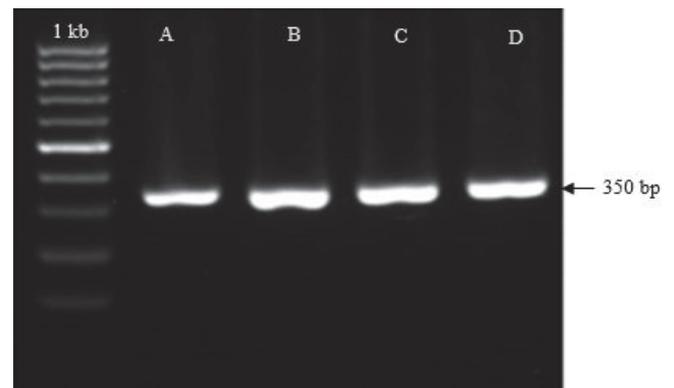


Fig.1. Electrophoresis of PCR products of ABC2 Transporter gene in *Trypanosoma evansi* A (Placebo), B (ISM 0.05 mg/kg weight), C (ISM 0.1 mg/kg weight) or D (ISM 0.3 mg/kg weight), showed 350 basepairs.

Nucleotide substitution mutations are divided into three types in the form of synonymous subtitles, nonsynonymous subtitles, and nonsense subtitles (Saito 2013). The base difference at site 107 in this study included mutations with the type of synonymous or silent mutations. These mutations are caused by changes in nucleotides but didn't change the function of amino acids (Figure 3). These are indicating that the ABC2 Transporter gene is conserved at the subspecies ABC transporter gene *T. brucei gambiense* (XM 011779815.1), *T. b. brucei* (XM 817911.1) and ABC Transporter 2 gene (Tbac2) in *T. brucei* (U89027.1) (Nuryadi et al. 2019). The results of the study that were translated into amino acids showed that all four samples had ABC signatures, namely LSGG (Figure 3). According to Klokouzas et al. (2003), the composition of the ABC transporter protein-coding gene is characterized by Walker A motif, ABC signature (LSGG), and Walker B motif. Exposure of *T. evansi* to ISM resulting no difference in nucleotide between *T. evansi* A, B, C, and D. Although *T. evansi* field isolates are suspected to be resistant to ISM, exposing animal models requires a long period around 3 -5 months. Furthermore, the immunity of mice can influence the process of drug resistance (Peregrine et al. 1997; Tihon et al. 2017).

The ABC2 transporter genes in *T. evansi* A, B, C, and D have a genetic distance close (0.003) to ABC Transporter putative gene *T. brucei gambiense* (XM 011779815.1), *T. b. brucei* (XM 817911.1), and ABC2 Transporter gene (Tbac2) in *T. brucei* (U89027.1) (table 1). The smaller genetic distance (closed to 0) shows a close genetically, while the greater genetic distance (Approaching to 1) shows a distant genetic relationship (Wuhan et al. 2020). Kaminsky (1998) shows the ABC transporter gene was identified as TbABC1, TbABC2, and TbABC3, which TbABC1 and TbABC2 had a homology with LtpgpA and Ldmdr1. Ldmdr1 is a gene that is related to drug resistance in *Leishmania donovani* (Hendrickson et al. 1993).

Nucleotide 41-80				
#T._evansi_A	TTCAGCTTGG	GGATGTGGAG	TACAGGCATC	AGTACCGCAA
#T._evansi_B
#T._evansi_C
#T._evansi_D
#XM_011779815.1_T._b._gambiense
#XM_817911.1_T._b._brucei
#U89027.1_Tbabc2_T._brucei
Nucleotide 81-120				
#T._evansi_A	GGTGATGGGT	TTTGTGGGTC	AAGATGACGT	GCTTTCAAAC
#T._evansi_B
#T._evansi_C
#T._evansi_D
#XM_011779815.1_T._b._gambiense	T
#XM_817911.1_T._b._brucei	T
#U89027.1_Tbabc2_T._brucei	T
Nucleotide 121-160				
#T._evansi_A	ATCTCAACGC	CGAAACGGTC	CCTCCGCTTT	TCTGTCCGCG
#T._evansi_B
#T._evansi_C
#T._evansi_D
#XM_011779815.1_T._b._gambiense
#XM_817911.1_T._b._brucei
#U89027.1_Tbabc2_T._brucei

Fig. 2. The result of ABC Transporter 2 Encoding Gene nucleotide sequences alignment analysis using ClustalW. The difference point is in base 107 of *T. evansi* A (Placebo), B (ISM 0.05 mg/kg weight), C (ISM 0.1 mg/kg weight), and D (ISM 0.3 mg/kg weight) (blue box).

Amino Acid 1-40				
#T._evansi_A	HLATSKDRKL	EGKIQLGDVE	YRHQYRKVMG	FVGQDDVLSN
#T._evansi_B
#T._evansi_C
#T._evansi_D
#XM_011779815.1_T._b._gambiense
#XM_817911.1_T._b._brucei
#U89027.1_Tbabc2_T._brucei
Amino Acid 41-80				
#T._evansi_A	ISTPKRSLRF	SVRVRNPDP	ETTKQVSDV	MDELGLQHCR
#T._evansi_B
#T._evansi_C
#T._evansi_D
#XM_011779815.1_T._b._gambiense
#XM_817911.1_T._b._brucei
#U89027.1_Tbabc2_T._brucei
Amino Acid 81-106				
#T._evansi_A	DTTVGTPGLV	AGLSGGERKR	CSMGVD	
#T._evansi_B	
#T._evansi_C	
#T._evansi_D	
#XM_011779815.1_T._b._gambiense	
#XM_817911.1_T._b._brucei	
#U89027.1_Tbabc2_T._brucei	

Fig. 3. The results of ABC2 Transporter Encoding amino acid sequence alignment analysis of, there are not amino acids in *T. evansi* A (Placebo), B (ISM 0.05 mg/kg weight), C (ISM 0.1 mg/kg weight) or D (ISM 0.3 mg/kg weight). The black box indicates synonymous substitution, while the red box indicates the ABC signature.

Based on the phylogenetic tree, *T. evansi* A, B, C, and D, are in the same clade with the ABC transporter gene *T. brucei gambiense* (XM 011779815.1), *T. b. brucei* (XM 817911.1), and ABC Transporter 2 gene (Tbabc2) in *T. brucei* (U89027.1), while the ABBCG1 gene *T. cruzi* (KP204022.1) and *ldmrd1* gene *L. donovani* (L06062.1) are in a different clade (Figure 4).

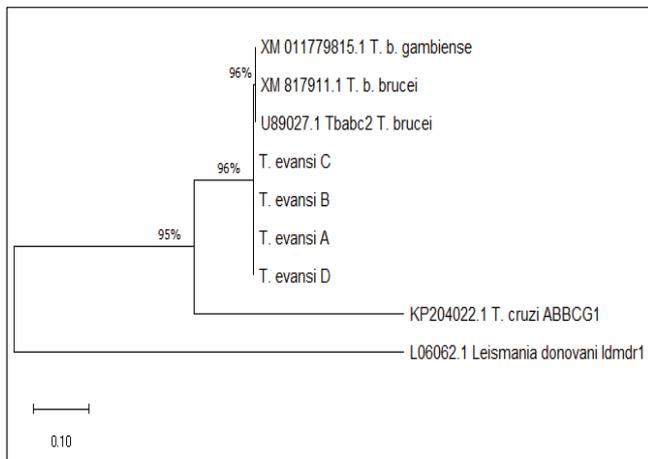


Fig.4. Phylogenetic of *Trypanosoma evansi* A (Placebo), B (ISM 0.05 mg/kg weight), C (ISM 0.1 mg/kg weight) or D (ISM 0.3 mg/kg weight) compared to other ABC Transporters (Gene Bank) using the Maximum Likelihood method.

4 Conclusion

The overall conclusion is that *T. evansi* which was exposed with ISM does not have many differences in nucleotide or amino acids and only has one type of mutation. The ABC2 Transporters of four groups of *T. evansi* have high similarity to ABC Transporters of *T. b. gambiense* (XM 011779815.1), *T. b. brucei* (XM 817911.1), and *T. b. brucei* (Tbabc2) (U89027.1). Therefore, further research on the ABC2 Transporter gene is needed.

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