IN VITRO DRUG SENSITIVITY OF *Trypanosoma evansi* BANGKALAN ISOLATES

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ABSTRACT

Only a limited number of drugs is available for Trypanosoma infection treatment. In Indonesia there are two chemoterapheutics (isometamidium and diminazene) used for treatment. The isometamidium does not act as a trypanocide of Indonesia isolates. However, the clinical action of diminazene to Trypanosoma has not been reported yet. The objective of this research was to obtain trypanocide concentration of Trypanosoma evansi Indonesia isolates by in vitro test. Drug sensitivities of Trypanosoma evansi Bangkalan isolates modified using Minimum Eagle Medium and HEPES medium were measured for diminazene aceturate and suramin using in vitro incorporation test. In control group, trypanosome inoculate was added as a placebo substance in microtiter plate containing trypanosomes. Bloodstream forms were isolated from Wistar strain Rattus norvegicus, incubated at room temperature in serial drug dilutions in a microtiter plate by adding 5 % of CO2. After 24 hours, the trypanosomes were counted by inverted microscope to determine trypanocide action. The two subjects research were different trypanocide action ($p<0.05$). The minimum concentration with trypanocide action of diminazene aceturate was found to be 8.480 $\mu g.ml^{-1}$.The minimum concentration with trypanocide action of suramin was 6.600 $\mu g.ml^{-1}$.

Keywords: *diminazene aceturate, suramin, Trypanosoma*

INTRODUCTION

African sleeping sickness and chagas diseases, which may be fatal if not treated, is caused by Trypanosome species. In veterinary, the trypanosomes case remains a problem and it can result in major scale economic decrease in farm industries. Only a limited number of drugs are available for the treatment of Trypanosoma species infection (Brun et al., 1989). The most commonly used ones are suramin for early stage and Mel B for advanced stages of the diseases. In veterinary the isometamidium chloride (Phenantridium) is also used to treat infection of *T. evansi*, but it needs a large dose to kill the parasites (Roostantia et al., 2003).

For CNS involvement in advanced stage, Mel B, as a part of DFMO new drugs, is predicted to be suitable in treating chronic patients (Van Niuwenhove, 1992). However in recent years, cases of drug failure with Mel B have been reported. Over 10 % of treated trypanosomes infection suffered relapses. Mel B resistant isolates of *T.B. gambiense* have thus far not been demonstrated in the field.

Diminazene aceturate (Berenil®) is a well-known kinetoplasticide that has been used extensively throughout the world. In recent years, most of the clinical trials published originated in Africa, where such infections are still common like a *T. congolense* (Gummow et al., 1995; Wahyudi et al., 2005). The clinical application of diminazene against *T. evansi* Indonesia isolated was not reported yet. In other side, the diminazene has a good prospect as a drug of choice against to *T. evansi* Indonesia isolates. For the first time, this study investigated trypanocide action of diminazene by in vitro test but not all techniques can be used in Indonesian conditions, although Croft et al., (1997) reported that various in vitro methods have been described for assessing the sensitivity of trypanosome populations to trypanocidal drugs.

In this report we described the antitrypanosomal effects of diminazene aceturate and suramin by simple in vitro technique for specific species of Indonesian veterinary trypanosomes as well as their trypanocide action for the leishmaniasis, chagas disease and african trypanosomes. The objectives of this study were:

1. To obtain trypanocide phenomenon of diminazene and suramin against local trypanosomes isolates (*T. evansi* Bangkalan isolates)
2. To obtain of trypanocide concentration of diminazene and suramin by in vitro test against local trypanosomes isolates (*T. evansi* Bangkalan isolates).

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MATERIALS AND METHOD

Trypanosome strains

T. evansi strains used were Bangkalan isolates obtained from The Veterinary Research Institute, RE Martadinata 65, Bogor, type of culture collection: BAKIT 87/649.

Drugs

Diminazene aceturate was obtained from Hoechst Pharmaceutic Industries, Netherlands by PT Intervet-Indonesia. Suramin was obtained from branch office of Bayer Pharmaceutical Industries in Surabaya, Indonesia (PT Bayer-Indonesia). The diminazene aceturate and suramin are dissolved in DMSO at 10 mg/ml if necessary, and then heated and/or sonicated. If insoluble, other solvents were used according to recommendations of the supplier. The compounds were produced to 25 ppm and 90 ppm as stock solution. The stocks were kept at 4^0C for a maximum of 2 weeks, and, thereafter at – 20^0C. For the assay, fresh dilutions in medium were prepared each time. For control, the phosphate buffer glucose (PBG) of the parasites stability was used as a placebo compound.

In vitro growth inhibition studies

Bloodstream trypomastigote form were cultured in Minimum Essential Eagle Medium with Earle salt + 25 mM HEPES + 1g/L glucose for molecular biology + 10 mM Minimum Essentiale Eagle Medium with Earle salt and sod. bicarbonate without non-essential amino acid + 0.2 mM 2 –mercaptoethanol + 2 mM Sod. piruvic acid, + 0.1 mM Hipoxanthine +15 % horse serum (in active).

Animals

Male Wistar strain *Rattus norvegicus* (200 g to 250 g) were purchased from Pusat Veterinaria Farma, the Directorate General of Livestock Services, Ministry of Agriculture, Jalan Ahmad Yani , Surabaya.

Separation trypanosomes from blood infected rats

The trypanosomes strains were maintained and propagated in Wistar strain *Rattus novegicus* by intraperitoneal inoculation of infected blood. After 5-6 days post inoculation, the rat had clinical condition of parasitemic 4^{th} positive (Lazuardi, 1998). For fractionation, the animals were exsanguinated under relaxation without anaesthetic agent with heparin (10 IU/ml of blood) as an anticoagulant. In most infections (4^{th} parasitemic stages) the animals were bled 4-5 ml by cardiac puncture at about 5-6 days after inoculation. The blood was kept in ice, diluted 1 : 3 with phosphate buffer glucose and filtered through gauze to remove any small clots or debris. Fractionation was started as soon as possible (Lanham, 1968).

Buffers were prepared from AnalaR grade reagents except for tris which was technical grade. Phosphate saline-glucose buffer (PSG) pH 8 consisted of 285 ml of 0.2 M Na2HPO4, 15 ml of 0.2 M Na2H2PO4, 300 ml of 0.85 % (w/v) NaCL and 400 ml of 2.5 % (w/v) glucose. Tris-saline glucose buffer (TSG) pH 7.8 consisted of 5.8 ml of 1 M tris, 40 ml of 1 N HCL, 222 ml of H2O, 200 ml of 0.95 % (w/v) NaCL and 4.5 g of glucose. The anion-exchanger DEAE-cellulose (Type DE 52, whatman Chromedia) in conjunction with PSG gave the best yields of all the trypanosomes.

The DEAE- cellulose was equilibrated with batches of buffer in the ratio of 100 g of pre-swollen (DE 52) adsorbent to 1.5 L. The adsorbent was allowed to settle for about 20 minutes and the supernatant containing the fines was sucked off. This was repeated five or six times until the pH of the supernatant was within 0.05 units of the buffer pH. Heparin (10 IU) from a solution of 500 IU/ml, in 0.95 % (w/v) NaCL was added to each ml of slurry after the final wash. The equilibrated slurry could be stored at – 10 ^{0}C. When required it was thawed, resuspended in 5 volumes of fresh buffer and any further fines removed. For sterile fractionation, the adsorbent was autoclaved at 121^0C for 15 minutes, either in the initial pre-swollen form or in aqueous suspension, and the equilibrated with buffer sterilized by Seitz filtration.

The preparation of the column and the fractionation of the blood were both carried out at room temperature, although the unprocessed blood and eluate were kept in ice baths. The one g of dry DEAE-cellulose (4 g of pre-swollen DE 52) was equilibrated with PSG adsorbent 1-1.6 ml of blood, but when equilibrated with TSG only 0.8-1 ml of blood was absorbed. A 20 % excess of adsorbent was found to be about 10 % less efficient. Columns ranging from 0.4-2.5 cm in diameter by 4-8 cm in height were satisfactory for fractionating up to 5 ml of blood. With large volumes the diameter of the column was increased relative to the height to maintain a fast rate of flow. The column was produced from disposable injection 10 ml without pestile with adding sterilized cotton wool at the outlet of disposable injection (porosity about 0.025 or 0.05 mm).

To pack the column, the DEAE on the disposable injection was pressed slowly until the density of DEAE is quite compact. The DEAE was moistened with buffer and the slurry run in. The excess liquid was run out and eluting buffer then run through the column to bed down the adsorbent with a firm horizontal surface. The outlet

was closed and cold diluted blood carefully layered on to the surface. A rate of flow was maintained using gentle suction when necessary, which gave a sharp descending front of erythrocytes. When all the blood had entered the adsorbent buffer was run through to column until microscopic examination showed that few or no trypanosomes were still being eluted. The trypanosomes were separated from the eluate by centrifugation at 2 ^{0}C at 1.630 g followed by repeated washing with buffer to remove the plasma proteins (Lazuardi, 2004).

Assay Procedure (Brun et al., 1996; Lazuardi, 2004)

1. The cover of plate 96 well was marking with sign pen as follows :

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Diminazene aceturate				Suramin				Placebo			
C												
D												
E												
F												
G												
H												

2. A 100 µl of medium warmed to room temperature are added to each well of a 96-well Costar plate in rows A to G, Row H is left empty.
3. A 50 µl of medium, containing two-times the highest drug concentration, was added to the wells of row H. Three compounds can be tested this way on each plate (drug 1 columns 1-3, drug 2 columns 5-7, placebo 3 column 9-11).
4. Serial drug dilutions were prepared with a multipipette 25 µl, taken from wells of row H and transferred into wells of row G. After mixing, 25 µl was transferred from wells of row G to wells of row F and so forth to row B. The 25 µl removed from wells of row B was discarded. A serial dilution factor of 1:3 was thus obtained. The drug concentrations ranged from 90 µg/ml to 0.37 µg/ml. For active compounds the highest concentration was appropriately lowered. Wells of row A served as control without drug.
5. A 100 µl of medium without trypanosomes was to columns 4.8 and 12 wells B to H which served as positive controls.
6. A 50 µl of a trypanosome suspension was added to all remaining wells containing $\geq 3.10^2$ bloodstream forms leading to a initial trypanosome density of 3 x 10^3.ml^{-1}.
7. The plates were incubated at 37 ^{0}C in a humidified atmosphere containing 5 % CO2.
8. After 24 hours the plate was inspected under an inverted microscope to assure growth of the controls and sterility of plate, and to determine the minimum trypanocide concentration by definition of test score :
   (-) : No body trypanosomes still life on the well
   (+) : Minimum one trypanosomes still life on the well
9. After obtained trypanocide concentration, a new serial drugs had to be made. The serial drug interval of diminazene produced was about 1 ppm concentration. The new serial of suramin was produced in a concentration range of 0.01 ppm to 10 ppm (Lazuardi, 2004). The application method of new serial drug assay was the modification of the method by Roostantia et al. (2003).

Statistic analysis

Statistic analysis was performed using by SPSS 11.0 at fisher exact test significancy of 0.05 between data from trypanocide concentration of diminazene vs. suramin and diminazene vs. control.

RESULTS

The in vitro drug response of *Trypanosoma evansi* Bangkalan isolates from horse and bovine outbreak diseases in Bangkalan 1988 to diminazene and suramin was examined and shown in Table 1 and Table 2. For diminazene the trypanocide values ranged from 3.330 µg/ml to 10.000 µg/ml of the stock 90 ppm and ranged from 3.300 µg/ml to 9.900 µg/ml of the stock 25 ppm (table 1). More analysis in trypanocide action of diminazene showed a range from 8.480 µg/ml to 9.540 µg/ml (table 2). For suramin, the trypanocide values ranged from 3.300 µg/ml to 6.600 µg/ml (table 2). Figure 1 showed two bodies trypanosomes of the 8.48 µg/ml diminazene treated wells.

Table 1. The ranged trypanocide concentration of diminazene

Dim. acet. from stock I (μg/ml)	Wells examined dim. acet from stock I			Dim. acet. from stock II (μg/ml)	Wells examined dim. acet from stock II			The placebo compund (Control)		
90.000	-	-	-	29.700	-	-	-	+	+	+
30.000	-	-	-	9.900	-	-	-	+	+	+
10.000	-	-	-	3.300	+	+	+	+	+	+
3.330	+	+	+	1.100	+	+	+	+	+	+
1.110	+	+	+	0.370	+	+	+	+	+	+
0.370	+	+	+	0.120	+	+	+	+	+	+
0.111	+	+	+	0.090	+	+	+	+	+	+

- : No body trypanosome still alive
+ : Any body trypanosomes still alive

Table 2. The ranged trypanocide concentrations of diminazene and suramin

Dim. acet. (μg/ml)	Wells examined of dim. acet			Suramin (μg/ml)	Wells examined of suramin			Placebo (Control)		
31.730	$-^a$	$-^a$	$-^a$	9.900	$-^b$	$-^b$	$-^b$	$+^c$	$+^c$	$+^c$
12.720	$-^a$	$-^a$	$-^a$	6.600	$-^b$	$-^b$	$-^b$	$+^c$	$+^c$	$+^c$
10.577	$-^a$	$-^a$	$-^a$	3.300	$+^b$	$+^b$	$+^b$	$+^c$	$+^c$	$+^c$
9.540	$-^a$	$-^a$	$-^a$	1.100	$+^b$	$+^b$	$+^b$	$+^c$	$+^c$	$+^c$
8.480	$-^a$	$-^a$	$+^a$	0.370	$+^b$	$+^b$	$+^b$	$+^c$	$+^c$	$+^c$
7.420	$+^a$	$+^a$	$+^a$	0.120	$+^b$	$+^b$	$+^b$	$+^c$	$+^c$	$+^c$
6.360	$+^a$	$+^a$	$+^a$	0.090	$+^b$	$+^b$	$+^b$	$+^c$	$+^c$	$+^c$
5.300	$+^a$	$+^a$	$+^a$	0.030	$+^b$	$+^b$	$+^b$	$+^c$	$+^c$	$+^c$
4.240	$+^a$	$+^a$	$+^a$	0.020	$+^b$	$+^b$	$+^b$	$+^c$	$+^c$	$+^c$
3.530	$+^a$	$+^a$	$+^a$	0.010	$+^b$	$+^b$	$+^b$	$+^c$	$+^c$	$+^c$

- : No body trypanosome still life
+ : Any body trypanosomes still life
The different of superscript at the same rows was different $p < 0.05$

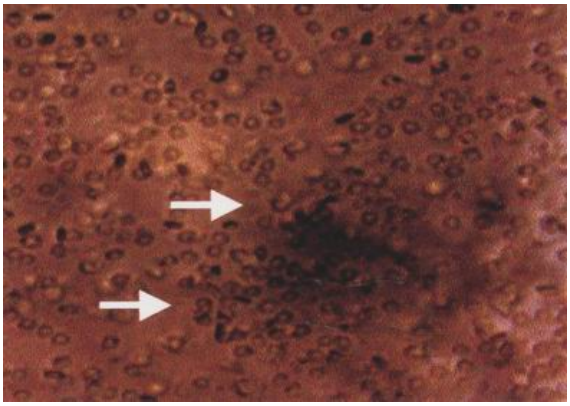

Figure 1. The trypanocide concentration of diminazene aceturate at 8.48 ug/ml to *T. evansi* Bangkalan isolates examined in microtiter plate well by inverted microscope.

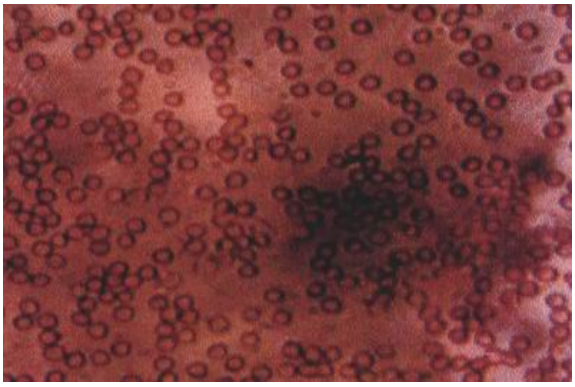

Figure 2. The trypanocide concentration of suramin at 6.60 ug/ml to *T. evansi* Bangkalan isolates examined in microtiter plate well by inverted microscope.

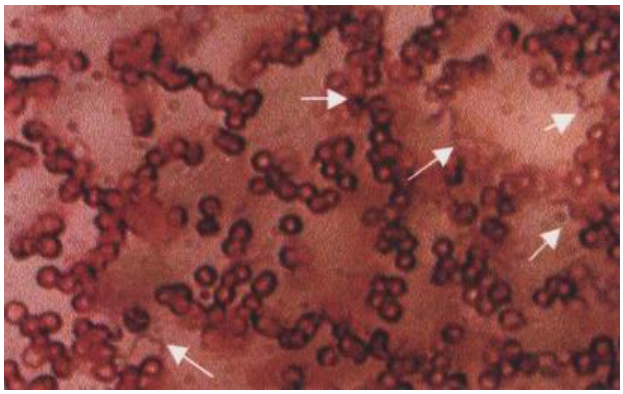

Figure 3. The placebo compound to *T. evansi* Bangkalan isolates examined in microtiter plate well by inverted microscope.

Figure 2 showed no body trypanosomes in microtiter plate wells of the treated drugs but Figure 3 showed much trypanosomes still lives in microtiterplate of the placebo control. The medium of trypanosome as a stabilat compound (PSG) was shown already steril condition and did not find the mold or other microorganism.

The result of the analysis statistic was shown that suramin significantly efffective greather than diminazene ($p<0.05$). Trypanocide concentration of the diminazene finding at 8.480 μg/ml, but the concentration trypanocide of suramin obtained lowest than diminazene concentration (6.600 μg/ml). More than 6.6 μg/ml of the suramin showed very effective to *Typanosomes evansi* Bangkalan isolate.

The placebo compound as a research control showed much living trypanosomes on the microtiter plate wells (Figure 3), but the amount of the trypanosomes decreased after incubation more than two or three days, although CO2 and temperature were at the appropriate condition.

DISCUSSION

The sensitivity of *Trypanosoma evansi* Bangkalan isolates to the trypanocydal drugs (diminzene aceturate and suramin) was accurately determined in an in vitro assay examined by inverted microscope. The findings demonstrated that drug potency and resistance can be detected using in vitro system as mentioned by Kaminsky and Zweygarth (1989). It was highly difficult for predicting drug potency to trypanosomes isolates using bioassay method based on immunodiagnostic assay. Solihat et al. (1996) illustrated that immunodiagnostic assay for testing potency of the trypanocide drug usually had problems of false positive or false negative conclusions.

These results indicated that complexion of parasites with diminazene in *Trypanosome evansi* Bangkalan isolates inhibited the capacity of trypanosomes to conduct biosynthesis effectively. However, that action apparently lowered suramin (Table 2). Unfortunately, suramin, as a drug of choice for *T. evansi* Indonesia isolates since 1998, was not produced as a trypanocide drug.

The trypanocide concentration of diminazene was obtained at 8.480 μg/ml, lower than isometamidium chloride (11.200 μg/ml) to *T. evansi* Bangkalan isolates as mentioned by Roostantia et al., (2003). That phenomena showed that, after its dose being re-arranged, diminazene would be a reliable drug of choice to trypanosomes Indonesia isolates. The effective concentration of diminazene for field isolates should be given approximately more than 8.48 μg/ml. The concentration of diminazene lowest than available concentration (8.480 μg/ml) reduced sensitivities to the drug. This reduced in vitro sensitivity is reflected to in vivo results. The non responsiveness of the diminazene is a serious drawback for the potential development of diminazene for treatment of trypanosomiasis in Indonesia. Because drug resistance is a major problems in chemotherapy of livestock and human trypanosomiasis as mentioned by Kaminsky and Brun (1998).

CONCLUSION

The diminazene aceturate and suramin have a potency therapy for *T. evansi* Bangkalan isolates at 8.480 μg/ml and 6.600 μg/ml as a available concentration.

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