J. Protozool. Res., 8, 241-248 (1998)
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Axenic Culture of *Trypanosoma evansi:* An Application to the Simple Detection of Sensitivity of Bloodstream Trypomastigotes to Trypanocidal Drugs

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Received August 20, 1998

Key words: axenic culture, diminazene aceturate, drug sensitivity test, *Trypanosoma evensi*

ABSTRACT

An attempt to the development of a simple in vitro method to detect the drug sensitivity of *Trypanosoma evansi* was made. Bloodstream forms (BSFs) of kinetoplastic *T. evensi, T. evansi* IL3354 and *T. evensi* IL3960 populations, and akinetoplastic *T. evensi* AK, *T. evensi* IL1934 and *T. evensi* IL3960-AK populations were propagated in vitro in an axenic culture system, in which bloodstream forms of *T. evansi* were cultured in the absence of feeder layer cells using HMI-9 culture medium following the procedure described earlier (Hirumi and Hirumi 1994).

With a minor modification of the original drug sensitivity test (Hirumi et al. 1993), the minimum effective concentration of diminazene aceturate (DA) (Berenil® and TRYPAN®) was tested for the *T. evensi* populations. Five hundred μl aliquots of trypanosome suspension in the medium, containing 2 x 10^4 BSFs, were placed in each well, added an equal volume of drug solution containing various concentrations of DA in culture medium and maintained at 37℃ with 5% CO_2 in air for 10 days without medium change. Effects of the drug were examined by phase-contrast microscopy every 24 hrs. In the control group with no drug, trypanosomes increased in number during the initial 3 days and reached the maximum cell density (1-2 x 10^6 BSFs/ml). Trypanosomes, thereafter, rapidly decreased during the following 2 days and died by day 7 due to overpopulation. In contrast, trypanosomes maintained in the medium contained ineffective doses of DA, rapidly decreased in number and died by days 3-5. While, in the medium contained lower doses, numbers of BSFs gradually decreased to the levels of 10-10^3 BSFs/well during the initial 3-5 days. Thereafter, the survived parasites increased in number reaching the maximum density during the following 3-5 days and then died by days 7-10.

Although no significant difference was observed between the kinetoplastic and chemically induced akinetoplastic populations tested, the results demonstrated that the minimum effective concentration of the drug tested can be readily detected by means of phase-contrast microscopy without aids of sophisticated equipments, including a freeze-dryer which was used in the original method. The method may be simple enough to apply in ordinary research laboratories which are equipped with standard cell culture equipments including a CO_2 incubator and an inverted phase-contrast microscope. The results were highly reproducible.

INTRODUCTION

The development of in vitro technology for culturing Salivarian trypanosomes may be divided into four phases. Phase I, from 1903 to 1975, during this period only procyclic

forms were continuously cultured. Phase II, from 1976 to 1988, basic methods were established to culture all the developmental stages of *Trypanosoma brucei brucei, T. b. gambiense, T. b. rhodesiense, T. congolense* and *T. vivax*. Phase III, from 1985 to 1993, during this period further advancements were made for propagating BSFs in axenic culture systems. Bloodstream forms of all the major pathogenic species of Salivarian trypanosomes, including *T. evansi,* can now be cultured in vitro in the absence of feeder layer cells (Hirumi and Hirumi 1994, also see the paper by Zweygarth in this proceedings). Phase IV, since 1993 to date, a number of attempts to develop a serum protein-free medium which would be the ultimate medium for culturing Salivarian trypanosomes has been made. Recently the first step toward the establishment of such a system was reported (Hirumi et al.1997).

At present, chemotherapy and chemoprophylaxis are still the main methods for controlling the diseases (Kaminsky 1990). However, the extensive use of a limited number of commercially available trypanocides during the last several decades, particularly for the control of bovine trypanosomosis, has resulted in the appearance of drug-resistant parasites in many parts of Africa (Leach and Roberts 1981, Pinder and Authié 1984). Improved surveillance for drug resistance encountered in the field is essential for more efficient control of the diseases (Kaminsky 1990) since, in some situations, the resistance has become so prevalent that the drugs concerned have been withdrawn from general use (Leach and Roberts 1981).

The standard method which is still widely in use for assessment of drug resistance is testing of the susceptibility of trypanosomes to trypanocidal drugs in experimental animals (Kaminsky 1990). These tests often require a number of animals and are time-consuming, in general taking 30-60 days. Thus, simple but reliable alternative methods for detecting the sensitivity of trypanosomes to the drugs in vitro are highly desirable.

The establishment of in vitro methods for cultivating BSFs of *T. b. brucei* (Hirumi et al. 1977), *T. congolense* (Hirumi and Hirumi 1984) and *T. vivax* (Brun and Moloo 1982, Hirumi et al. 1983) in the presence of mammalian feeder layer-cells has led to the development of in vitro systems for determining drug sensitivity (Barowy et al. 1985a, 1985b, 1985c, 1988, Ross and Tailor 1990, Zweygarth and Kaminsky 1990). However, the requirement for feeder layer-cells in these culture systems has hampered their use in studying trypanocidal activities in vitro (Kaminsky 1990). This problem has recently been overcome by the development of feeder layer-free culture systems for BSFs of *T. brucei* (Baltz et al. 1985, Duszenko et al. 1985, Hirumi and Hirumi 1989), *T. congolense* (Hirumi and Hirumi 1991), *T. simiae* (Zweygarth et al. 1992) and *T. vivax* (Hirumi et al. 199la, Zweygarth et al. 1991a).

Following the introduction of these axenic culture systems, considerable progress in the in vitro assessment of trypanocidal activities of various compounds in BSFs of *T. brucei* (Bouteille et al. 1988, Brun and Kunz 1989, Brun et al. 1989, Kaminsky and Zweygarth 1989a, 1989b, 1991, Kaminsky et al. 1989, Zinsstag et al. 1991a, Zweygarth and Kaminsky 1990, 1991, Zweygarth et al. 1990) and, to a limited extent, in *T. vivax* (Zweygarth et al. 1991b) has been made. In *T. congolense*, in vitro trypanocidal activities have been examined by using the insect-vector stage (procyclic forms) (Brun and Rab 1991, Elrayah and Kaminsky 1991, Ross and Tailor 1990), and by the incorporation of [H]-hypoxanthine in short-term BSFs culture systems in the absence (Brun and Rab 1991) or the presence (Ross and Tailor 1990) of feeder layer-cells. A simple method for the in vitro detection of sensitivity levels to 4 trypanocidal drugs which are commonly in use for BSFs of *T.*

congolense has also been developed (Hirumi et al. 1993). In the present study, an attempt to simplify further this method has been made for *T. evansi*.

MATERIALS AND METHODS

Trypanosomes. Three kinetoplastic (K) and 3 akinetoplastic (AK) strains of *T. evansi* listed in Table 1 were tested .

Table 1. List of the *Trypanosoma evansi* strains tested.

Strain	Kinetoplast	Isolation		
		Host	Place	Year
T. evansi Tansui*	K	Water buffalo	Taiwan	NR
T. evansi Tansui-AK*	AK †	PR-induced AK	NITM	1996
T. evansi IL1934**	AK §	Capybara	South America	1971
T. evansi IL3354**	K	Camel	Mali	1988
T. evansi IL3960**	K	Camel	Kenya	1980
T. evansi IL3960-AK	AK #	PR-induced AK	RCPMI	1998

* Obtained from Prof. H. Kanbara, the Inst. Trop. Med., Nagasaki Univ. (NITM), Nagasaki, Japan (Silva-Thant et al. 1997).
** Obtained from ILRI (former ILRAD), Nairobi, Kenya.
† Akinetoplastic strain induced by pararosaniline (PR) treatment in mice at the NITM (Silva-Thant et al. 1997).
§ Naturaly kinetoplastic strain.
\# Akinetoplastic strain induced by PR treatment in culture at the Obihiro Res. Cent. Protozoan. Mol. Immunol.
NR: No record was available.

Bloodstream forms of each strain were isolated from infected BALB/c mice, separated from the blood cells by means of DE-52 cellulose chromatography (Lanham and Godfrey 1970) and propagated in axenic culture following the procedure reviewed earlier (Hirumi and Hirumi 1994) using HMI-9 medium that has been originally developed for the axenic culture of *T. brucei* (Hirumi and Hirumi 1989).When the cultures became stable showing consistent growth rates, BSFs of each strain collected by centrifugation at 3,000*g* for 15 min. and resuspended in fresh medium at 2 x 10^4 BSFs/ml. Five hundred μl of the trypanosome suspension in the medium were placed in each well of 24-well culture plates (Falcon 3047, Becton Dickinson, N.J., USA).

Trypanocidal drug. Two sources of diminazene aceturate were used; Berenil®, powder form (Hoechst AG, Germany) and TRYPAN®, liquid form (Dipl. Inst. Sup. Tech-Dev. Med., Hamburg, Germany; see the paper by Bourdichon in this proceedings for the details). The first DA stock solutions of Berenil and TRYPAN were prepared in 10 ml of distilled water containing the same concentration of the active gradient of DA, then test solutions were prepared by 10-fold (for high concentrations) and 2-fold serial dilutions (for the final test solutions) in culture medium. Five hundred μl of the test solutions containing 2 times concentrations of the final testing doses of DA was added to each well that received 500 μl of the trypanosome suspension (final seeding density: 1 x 10^4 BSFs/ml/well). The final concentrations of DA tested were 640, 320, 160, 80, 40, 20, 10. 5 and 0 ng/ml.

Detection of the minimum effective dose. The minimum effective dose was detected by maintaining the test plats at 37℃ with 5% CO_2 in air for 10 days without medium change. And effects of the drug on the growth of *T. evensi* BSFs were evaluated by means of

phase-contrast microscopy every 24 hrs.

RESULTS

Prior to the final tests, the effectiveness of Berenil and TRYPAN on the growth of *T. evansi* IL3354 BSFs, which had been well adapted to in vitro culture for more than 10 months and exhibited a constant growth rate, were compared. This preliminary test indicated that there was no detectable differences regarding the degree of growth inhibition between the two drugs. Thus, all remaining tests were made using TRYPAN since its liquid form made the preparation of test solutions easy.

The results of tests (each test was made in 2 wells/a drug concentration; each test was repeated 3-6 times; Tansui: 6 times, Tansui-AK: 6 times, IL1934: 4 times, IL3354: 3 times, IL3960: 4 times, 3960-AK: 4 times). Since the results of 6 repeated tests on *T. evansi* Tansui and Tansui-AK were highly consistent, remaining tests were repeated 3-4 times.

In control (without drug), trypanosomes increased in number during the initial 3 days and reached the maximum cell density (1-2 x 10^6 BSFs/ml), thereafter rapidly decreased during the following 2 days and died due to the overpopulation by day 7. In such wells the color of medium changed slightly yellowish (amber), indicating pH 6.8-7.0.

In the wells contained effective concentrations of DA, trypanosomes rapidly decreased in number showing no increase in number from the seeding density and died by days 3-5. The color of the medium in these cultures showed slightly pinkish (pH 7.8 ±). This colorimetric change was not as distinct as that seen in the affected test wells of *T. congolense* BFSs (Hirumi et al. 1993).

In contrast, test wells contained ineffective doses of DA, two types of growth patterns were observed. Type I: the growth pattern was similar to that seen in the control group. This type was observed in wells contained low doses of DA as expected. Type II: in the wells contained higher doses, but lower than the effective doses, numbers of trypanosomes gradually decreased to the levels of 10-10^3 BSFs/well (depending on the DA concentrations) during the initial 3-5 days. Thereafter the survived parasites increased in number reaching the maximum density during the following 3-5 days and then died due to overpopulation by days 7-10.

The results of the tests are summarized in Table 2.

Table 2. Growth inhibition of *T. evansi* BSFs at various concentrations of diminazene aceturate (DA)* in culture.

Strain	Growth inhibition								
	DA concentration (ng/ml)								
	640	320	160	80	40	20	10	5	0
T. evansi Tansui	+	+	+	-	-	-	-		-
T. evansi Tansui-AK	+	+	+	-	-	-	-		-
T. evansi IL1934	+	+	-	-	-	-	-		-
T. evansi IL3354		+	+	+	+	-	-	-	-
T. evansi IL3960			+	+	+	-	-	-	-
T. evansi IL3960-AK			+	+	+	-	-	-	-

*TRYPAN

DISCUSSION

Although there was no distinctive difference between kinetoplastic strains (Tansui

and IL3960) and their chemically induced akinetoplastic strains (Tansui-AK and IL3960-AK, respectively), the minimum effective concentrations of DA for Tansui, Tansui-AK and IL1934 (160 ng/ml) were 4 times higher than that of IL3354, IL3960 and IL3960-AK (40 ng/ml). These results suggest (1) that kinetoplasts (minicircle DNA) of *T. evansi* may not involve with trypanocidal activities of DA and (2) that great differences regarding the sensitivities to DA may exist widely among different populations of *T. evansi*, suggesting the need of precaution in determining the optimum dose of DA for field treatments.

The Type II growth pattern seen in the wells contained unaffected doses of DA suggests that the trypanosome population was a mixed population consisted of parasites which were "sensitive" and "resistant" to the the drug at the dose tested.

In drug sensitivity tests it is essential to detect the presence of such parasites that are resistant to a given concentration of trypanocidal drug, even in a small number, since such parasites would be responsible for the relapse of the infection after the treatment. In this study, therefore, the lowest concentration which inhibited the growth of "all" parasites was considered to be the minimum effective dose.

In the axenic culture system applied in the present study, a small number of *T. evansi* BSFs (even as small as 10 out of 10^4 BFSs/well) is able to continue to multiply without medium change in the presence of large numbers of dead trypanosomes (sensitive) for at least 10 days or until they reach the maximum population density. This ability of the system to support the continuous growth of a small number of the BSFs without medium change for 10 days is particularly advantageous in detecting the minimum effective dose, since it enables the system to selects for a small number of unaffected (resistant) trypanosomes from the majority which are sensitive to a given concentration of test drug.

The maintenance of the axenic cultures is simple, although certain skills may be still required to initiate the culture from the original isolates and/or laboratory stocks which have not been adapted to in vitro culture (Hirumi and Hirumi 1991).

Another advantage of the in vitro assay described here is the requirement for only basic laboratory equipments for cell culture, such as a CO_2 incubator and an inverted phase-contrast microscope (Hirumi et al. 1993), and no requirement for sophisticate equipments, such as a Spectrophotomer, Enzyme-linked Immunosorbent Assay (ELISA) Reader, Liquid Scintillation Counter and a Coulter Cell Counter, used in earlier work (Brun and Kunz 1989, Brun and Rab 1991, Kaminsky and Zweygarth 1989b, Kaminsky et al. 1989, Ross and Tailor 1990, Zinsstag et al. 1991) but hardly available in many laboratories in developing countries.

In the original method (Hirumi et al. 1993), all test plates were prepared by the freeze-dry method which considerably simplified the preparatory procedure of the test plates. The freeze-dried test plates were wrapped with aluminum foil and could be stored at room temperature at least for 6 months without any detectable deterioration of the trypanocide's efficacy. However, in this study, the step of the freeze-dry was omitted with the consideration of unavailability of a freeze-dryer in many laboratories, particularly in the developing countries. Thus, the test plates were prepared prior to each test in the present study.

In the original method for the tests on *T. congolense* BSFs, the minimum effective dose was detected based on the colorimetric reaction of the culture medium. The color of the medium in affected cultures changed to pinkish color ($pH>7.8$) which was highly distinctive from the yellowish medium ($pH\leqq6.0$) in unaffected cultures. In such unaffected cultures,

changes in *T. evansi* BSF cultures were less distinct (amber) than that observed earlier in the test cultures of *T. congolense* BSFs and somewhat similar to that observed in the test cultures of *T. brucei* (Hirumi et al. 1993).

This may be due to the difference of the maximum population densities between BSFs of *T. congolense* and those of *T. evansi*, obtained under the culture conditions used. Bloodstream forms of *T. congolense* attach to the bottom surface of culture vessels, rapidly multiply and reach the maximum population density at the level of >5 x 10^6 BSFs/ml without medium change, resulting in the color change of the medium to the distinct yellow. In contrast, BSFs of *T. evansi*, like *T. b. brucei*, are freely "swimming" in the medium and reach the maximum population density at the level of 1 x 10^6 BSFs/ml resulting in the less distinctive color change, pH≒6.8-7.0. Thus, the final assessments of the effects of DA on *T. evansi* BSFs were made based on the direct observation of their growth by an inverted phase-contrast microscope.

The maintenance of the axenic culture system is also simple, although certain skills are still required to initiate the culture from the original isolates of *T. evansi* and/or from the laboratory stocks which have not been adapted to in vitro culture (Hirumi and Hirumi 1991).

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