J. Protozool. Res., 8, 233-240(1998)

In Vitro Cultivation of *Trypanosoma evansi*

E. ZWEYGARTH

Onderstepoort Veterinary Institute, Parasitology Division, Private Bag X5, Onderstepoort 0110, South Africa

Received 20 August 1998

INTRODUCTION

In recent years considerable progress has been made in the cultivation of cyclically transmissible stocks of trypanosomes, both procyclic and bloodstream forms, belonging to the subgenus *Trypanozoon*. In vitro propagation of trypanosomes has become a valuable tool for many aspects of research. In this paper, methods and techniques are described for the initiation and propagation of trypomastigote bloodstream form cultures of *Trypanosoma evansi*. The development of biphasic culture systems up to the stage of axenic propagation, as well as several applications, are presented.

FEEDER LAYER CELL CULTURES

Short-term cultivation of bloodstream forms of *T. brucei* in the presence of feeder layer cells was first reported by Le Page (1967). He used mouse L-cells and NCTC 109 medium. Under these condition, bloodstream forms were maintained for 3 days. It took another ten years before long-term cultures of *T. brucei* bloodstream forms were established (Hirumi et al. 1977). This pioneering work, using mammalian feeder layer cells, represented an immense breakthrough, and the new method was successfully extended from *T. brucei* to other members of the *Trypanozoon* group: *T. rhodesiense* (Hill et al. 1978), *T. gambiense* (Brun et al. 1981) as well as *T. evansi* (Zweygarth et al. 1982; Zweygarth et al. 1983).

FEEDER LAYER CELLS

Many different cell lines of various origins have been used as feeder layers for the in vitro cultivation of *T. evansi*. The first successful propagation of *T. evansi* was carried out in a biphasic culture system using chicken fibroblasts as a feeder layer (Zweygarth et al. 1982). This is the only report of bloodstream form trypanosomes which were propagated on feeder layer cells derived from a warmblooded, non-mammalian animal. Other cells which have been used include: rabbit fibroblasts (Zweygarth et al. 1983), pig fibroblastoid cells (Zweygarth and Röttcher 1986), bovine embryo thymus cells and bovine aorta endothelial cells (Zweygarth and Kaminsky 1990a), and mouse peritoneal macrophages (Baltz et al. 1985).

Initially, the exact function of feeder layer cells was not known. Bannai and Ishii (1980; 1982) found that human diploid fibroblasts take up L-cystine in the culture medium. In the cells L-cystine is immediately reduced to L-cysteine which is released back into the medium. Later we will see (in: "Axenic cultures") that trypanosomes cannot take up L-cystine but only L-cysteine, and that the latter is an essential amino acid for trypanosomes. Although it has not been specifically shown for trypanosome cultures, the principle described by Bannai and Ishii (1980; 1982) appears to be the rationale behind the necessity of using a feeder layer for trypanosome cultures. Any cell type releasing L-cysteine is potentially suitable as a feeder layer cell for *T. evansi* cultures, provided it does not compete for nutrients from the medium. A slow growing cell line is therefore preferable to a fast-growing line.

CULTURE MEDIA

Two culture media were used for the propagation of *T. evansi* in the presence of feeder layer cells: Roswell Park Memorial Institute medium 1640 (RPMI-1640) (Zweygarth et al. 1982; Zweygarth et al. 1983) and RPMI 1640 supplemented with 10% Leibovitz L-15 medium (Zweygarth and Röttcher 1986).

For axenic growth of *T. evansi* several other basic culture media were used: Eagle's minimum essential medium (Baltz et al. 1985; Kaminsky and Zweygarth 1989; Sutherland et al. 1993; Ross and Taylor 1994; Ross and Barns 1996), equal parts of Iscove's modified Dulbecco's medium and RPMI-1640, as well as Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (DMEM/F-12) (Zweygarth, unpublished results).

For axenic growth in serum-free medium, Hirumi et al. (1997) used an Iscove's modified DMEM-based medium. Bloodstream forms of *T. gambiense* and *T. rhodesiense* are incapable of de novo purine synthesis (Fish et al. 1982) and cultures of *T. evansi* require the medium to be supplemented with a nucleotide precursor, usually hypoxanthine. Baltz et al. (1985) found that, depending upon the source of serum, hypoxanthine was not always required: horse and rat serum had to be supplemented, whereas rabbit and human serum did not.

SERUM

Serum is an indispensable component for all media used for the in vitro propagation of *T. evansi*. It is the only semi-defned component of the culture media. Several animal sources of serum have been used successfully: horse, rabbit, rat, fetal bovine, and even fetal horse serum together with adult horse serum (Zweygarth et al. 1982; Zweygarth et al. 1983; Baltz et al. 1985; Sutherland et al. 1993). The concentration of serum in the complete culture media varied from 10% to 20%. Attempts to grow Kenyan stocks of *T. evansi* in medium containing samples of camel serum derived from different animals invariably failed to support in vitro propagation (Zweygarth, unpublished results).

Baltz et al. (1985) tried human serum, with negative results, which is not surprising. Hawking (1978) tested two *T. evansi* stabilates for their sensitivity to human plasma, both stocks were highly sensitive. Although bloodstream form trypanosomes can be propagated in medium containing fresh serum, it is advisable, especially in the presence of feeder layer cells, to inactivate the serum. Heterologous antibodies could negatively interfere with feeder layer cells, which in turn may have a negative impact on the survival of the trypanosomes. This reaction could be aggravated further by the presence of complement.

A serious disadvantage of the use of serum in the medium is its undefined nature. Batches of serum, even if obtained from the same donor animal, may vary considerably, so that laborious testing is necessary to identify suitable batches. Recently Hirumi et al. (1997) presented a serum-free medium for the in vitro propagation of *T. brucei* and *T. evansi* which could overcome these problems. However, although serum-free, it still contains semi-defined components such as bovine α_2-macroglobulin, bovine β-lipoprotein and fatty acid-free bovine serum albumin. Further experiments are needed in order to show whether the new medium can support growth of a wider variety of trypanosome stocks.

CULTURE ENVIROMENT

A temperature of 37℃ is required to propagate bloodstream forms of *T. evansi*, and a $C0_2$ atmosphere of 4 to 5% is usually used in order to control the pH of the culture medium. The candle jar method used for the propagation of *Plasmodium falciparum* (Trager and Jensen, 1976) was also used for the in vitro cultivation of African stocks of *T.*

evansi (Zweygarth and Röttcher, 1986). Culture plates containing the trypanosomes were put into a desiccator with a burning candle to produce a $C0_2$-enriched and 0_2-depleted atmosphere (this procedure was an improvisation and not due to a scientific curiosity or necessity, because at the time we had no $C0_2$ incubator).

INITIATION TECHNIQUES

The initiation of *T. evansi* bloodstream form cultures has been achieved after isolation of the parasite in an intermediate rodent host. Successful initiation of *T. evansi* cultures required a minimum inoculum of 5×10^3 or 1×10^5 trypanosomes/ml from mouse blood during a rising parasitaemia (Baltz et al. 1985; Zweygarth et al. 1982). The trypanosomes were separated from blood components either by density gradient centrifugation (Hirumi et al. 1977) or by difflerential centrifugation (Hill et al. 1978). These cumbersome procedures were later shown to be unnecessary by Zweygarth et al. (1989), who demonstrated a simple and rapid method for the initiation of bloodstream form cultures from infected blood avoiding both centrifugation and anion exchange procedures. Blood samples, for instance from the tip of the tail of an infected mouse, were aspirated with a 5 or 10 μl Eppendorf pipette and transferred into a well of a culture plate containing feeder layer cells. The blood was deposited in the corner at the bottom of the wells.

The low levels of parasitaemia usually present in natural hosts were initially considered insufficient to initiate cultures. However, trypanosome cultures were subsequently established regardless of the parasitaemic status of the blood inoculum and irrespective of whether the blood came directly from an animal or from a stabilate. Cultures of *T. evansi* were successfully initiated both under axenic conditions and in co-cultivation with a variety of feeder layers (Zweygarth and Kaminsky 1990a) using the method described above.

AXENIX CULTURE

Blitz et al. (1985) developed an anemic culture system which supported the continuous propagation of several members of the *Trypanozoon* group, including a clone of a South American *T. evansi*. They found that the presence in the medium of a reducing agent such as 2-mercaptoethanol (2-ME) or thioglycerol was crucial to the survival of the trypanosomes in anemic cultures. They assumed that the role of the reducing agent was to stabilize different essential components of the medium, such as vitamins and glutathione. In the same year Duszenko et al. (1985) found that *T. brucei* cultures were able to survive and grow without feeder layer cells if L-cysteine was added to the medium. Small amounts of L-cysteine had to be added to the cultures twice daily, depending on the trypanosome density. This laborious procedure is impractical for continuous propagation of parasites, but a solution was soon found to the problem.

L-cysteine is readily oxidize and has a half-life of about 2 h under culture conditions (Toothy et al. 1975). Copper ions, provided by the supplementation of the medium with serum, catalyse this reaction which produces hydrogen peroxide, which is toxic to trypanosomes. With mouse lymphoma cells, the addition to the culture medium of bathocuproine sulphurate (BAs), a specific copper ion chelator, prevented autoxidation of L-cysteine and thus promoted growth (Ishii and Bannai 1985). A similar approach was chosen by Yabu et al. (1989), who added BAs to the culture medium for *T. gambiense*, preventing the autoxidation and thus the trypanotoxic effect of L-cysteine. Hirumi and Hirumi (1989) added 2-ME as well as L-cysteine and BAs for the axenic propagation of *T. brucei*. Combinations of 2-ME, L-cysteine and BAs have been used for the in vitro

cultivation of *T. congolense*, *T. vivax* and *T. simiae* (Hirumi and Hirumi 1991; Zweygarth et al. 1991a and 1992) and also recently for the serum-free cultivation of *T. evansi* (Hirumi et al. 1997).

Duszenko et al. (1992) finally showed that cysteine is an essential growth factor for *T. bruvei* bloodstream forms, since trypanosomes cannot take up cysteine which is an essential amino acid. Cell growth was only obtained if cysteine was either added to medium or was reduced from cystine by the action of reducing agents.

The supplements of choice for several applications of the axenic in vitro culture technique of *T. evansi* were 2-ME or thioglycerol (Kaminsky and Zweygarth 1989; Zweygarth and Kaminsky 1990a; Sutherland et al. 1993; Hang et al. 1993; Fang et al. 1994; Ross and Tailor 1994; Ross and Barns 1996). A combination of L-cysteine and BAs alone is also a viable option (Zweygarth, unpublished results).

IIN VITRO DIFFERENTIATION BETWEEN *T. BRUCEI* AND *T. VEANSI*

The monomorphic *T. evansi* is morphologically indistinguishable from the long slender form of pleomorphic *T. brucei*. One of the main criteria to distinguish *T. evansi* from other members of the subgenus *Trypanozoon* is its inability to undergo a cycle of development in *Glossina*. This may be due to the fact that the kinetoplast DNA of *T. evansi* lacks maxicircles (Borst et al. 1987). Zweygarth and Kaminsky (1989) used a modified semi-defned maintenance medium (Cunningham 1977) and a suitable culture environment for culture experiments designed to allow bloodstream form trypanosomes to transform into procyclics. The assumption was that *T. evansi* cannot transform into procyclic forms, and it was found that all stocks previously identified as pleomorphic *T. brucei* by light microscopy of Giemsa-stained blood smears transformed into procyclic forms, while none of the *T. evansi* stocks did so. Olaho-Mukani et al. (1993) confirmed these observations with a greater number of parasite stocks. All the stocks they had previously classified as *T. rhodesiense*, *T. gambiense* and *T. brucei* transformed into procyclic forms, whereas none of *T. evansi* stocks did so. Three out of 64 field isolates from camels transformed into procyclics, confirming *T. brucei* infections in camels which were kept close to tsetse-infested areas.

LONG-TERM IN INVITRO CULTIVATION OF *T. EVANSI*

A stock of *T. evansi* was propagated in vitro for more than 14 months (Zweygarth et al. 1991b). After that period, a change of some biological properties was observed as compared to early cultures of the same stock. All organisms were dyskinetoplastic, probably because the shorter generation time of dyskinetoplastic forms led, after months of continuous propagation, to the disappearance of kinetoplastic forms. Simultaneously, a complete loss of infectivity for mice was observed, although an intact surface coat was demonstrated electron microscopically. The latter phenomenon remained unexplained.

The dyskinetoplastic *T. evansi* showed a reduced susceptibility to diminazene aceturate, which was probably a result of the preferential inhibition of extranuclear DNA synthesis which is a known feature of this trypanocidal drug (Newton and Le Page 1967). Drug susceptibility to isometamidium chloride, however, was unaltered. It is known that isometamidium chloride induces the destruction of the kinetoplast of *T. brucei* in vitro (Kaminsky et al. 1988) and interferes with topisomerase II in the kinetoplast, thus causing linearisation of DNA minicircles (Shapiro and Englund 1990). Since it was effective against dyskinetoplastic forms, it was concluded that isometamidium must have other targets for its

antitrypanocidal action. This was reconfirmed recently by Kaminsky et al. (1997).

ASSESSMENT OF DRUG SUSCEPTIBILITY

The introduction of axenic culture systems for the propagation of *T. evansi* (Baltz et al. 1985) opened up possibilities for the development of convenient in vitro drug screening systems. This is probably one of the most important applications of the culture system today. Kaminsky and Zweygarth (1989) described a feeder layer-free in vitro assay for the screening of antitrypanosomal compounds. Samples of trypanosomes in logarithmic growth phase were incubated with various concentrations of commercial and experimental compounds. Growth inhibition was monitored after 24 h and quantified by comparing the number of generations of controls and drug-treated cultures. This primary drug screening assay has a number of advantages: only small amounts of drug are required, results are obtained rapidly, and animals are unnecessary. Sutherland et al. (1993) introduced two in vitro systems for the measurement of drug sensitivity: incorporation of radio-labelled hypoxanthine and pyruvate production. Both were compared with assays dependent upon counting numbers of motile trypanosomes. They found that each assay system distinguished between suramin-sensitive and resistant stocks.

There have been several applications of these assays. A new arsenical compound (Cymelarsan) has been evaluated for its activity against susceptible and drug-resistant *T. evansi* (Zweygarth and Kaminsky 1990b); an in vitro determination of trypanocide resistance profiles has been performed (Zhang et al. 1993); the trypanocidal activity of a myristic acid analog has been estimated (Ross and Taylor 1994); and the alteration of an adenosine transporter which is associated with resistance to Cymelarsan has been demonstrated (Ross and Barns 1996). In a further development Fang et al. (1994) used in vitro cultures to induce suramin resistance in clones of *T. evansi*. By exposure to gradually increasing concentrations of suramin in an axenic culture system, suramin-resistant clones were derived from suramin-sensitive clones over a period of 550 days.

CONCLUSIONS

Stocks of *T. evansi* from all geographical regions where the disease is endemic were propagated in vitro. Initiation of cultures was achieved from blood with very low parasite counts and even from cryopreserved stabilates. An intermediate host to generate large numbers of parasites is therefore not absolutely necessary. *Trypanosoma evansi* needs a source of L-cysteine to survive in culture, which can be achieved by providing L-cystine and a suitable feeder layer which converts it to L-cysteine and secretes it into the culture medium. In a feeder layer-free system, L-cystine from the medium can be reduced to L-cysteine by the addition of reducing agents such as 2-ME or thioglycerol. Another possibility is to add L-cysteine directly to the medium together with the copper-chelating agent BCS which prevents its autoxidation.

The in vitro culture of trypanosomes offers a range of valuable applications, one of which is the in vitro transformation technique. This is a simple method to differentiate between *T. brucei* and *T. evansi* in regions where both species occur, although the method will not work with mixed infections. Since *T. evansi* does not undergo a developmental cycle in *Glossina* it will not grow in a culture system favouring the transformation of bloodstream forms to procyclics. Another organism which could be mistaken for *T. brucei* or *T. evansi* is *T. equiperdum*. However, its occurrence is restricted to equines, and may therefore be excluded by the case history. Furthermore, the parasitaemia is usually extremely low, so that

mechanical transmission of this species is very unlikely, although "stable infections" have been reported.

Another major area of application of culture techniques lies in the field of chemotherapy. The in vitro culture system offers a range of different tests for primary drug screening, for the establishment of drug susceptibility prohles of various stocks or clones, or even for the induction of drug resistance in vitro.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. B. A. Alsopp and Dr. D. T. de Waal for helpful comments on the manuscript.

REFERENCES

Baltz, T., Baltz, D., Giroud, C. & Crockett, J. 1985. Cultivation in a semi-defined medium of animal-infective forms of *Trypanosoma brucei, T. equiperdum, T. evansi, T. rhodesiense* and *T. gambiense. EMBO J. 4: 1273-1277.*

Bannai, S. & Ishii, T. 1980. Formation of sulfhydryl groups in the culture medium by human diploid fibroblasts. *J. Cell. Physiol. 104: 215-223.*

Bannai, S. & Ishii, T. 1982. Transport of cystine and cysteine and cell growth in cultured human dipoid fibroblasts: effect of glutamate and homocysteate. *J. Cell. Physiol. 122: 265-272.*

Brun, R., Jenni, L., Schbnenberger, M. & Schell, K.-F. 1981. In vitro cultivation of bloodstream forms of *Trypanosoma brucei, T. rhodesiense* and *T. gambiense. J. Protozool. 28: 470-479.*

Borst, P., Fase-Fowler, F. & Gibson, W.C. 1987. Kinetoplast DNA of *Trypanosoma evansi. Mol. Biochem. Parasitol. 23: 31 -38.*

Cunningham, I. 1977. New culture medium for maintenance of tsetse tissues and growth of trypanosomatids. *J. Protozool. 24: 325-329.*

Duszenko, M., Ferguson, M.A.J, Lamont, G.S., Rifkin, M.R. & Cross, G.A.M. 1985. Cysteine eliminates the feeder cell requirement for cultivation of *Trypanosoma brucei* bloodstream forms in vitro. *J. Exp. Med. 162: 1256-1263.*

Duszenko, M., MOhlstadt, K. & Broder, A. 1992. Cysteine is an essential growth factor for *Trypanosoma brucei* bloodstream forms. *Mol. Biochem. Parasitol. 50: 269-274.*

Fang, Y., Ye, W.X., Nei, H.Y. & Wang, Y.L. 1994. In vitro development of suramin-resistant clones of *Trypanosoma evansi. Acta Trop. 58: 79-83.*

Fish, W.R., Looker, D.L., Marr, J.J. & Berens, R.L. 1982. Purine metabolism in the bloodstream forms of *Trypanosoma gambiense* and *Trypanosoma rhodesiense. Biochim. Biophys. Acta 719: 223-231* .

Hawking, F. 1978. The resistance of *Trypanosoma congolense, T. vivax* and *T. evansi* to human plasma. *Trans. Roy. Soc. Trop. Med. Hyg. 72: 405-407.*

Hill, G.C., Shimer, S.P., Caughey, B. & Sauer, L.S. 1978. Growth of infective forms of *Trypanosoma rhodesiense* in vitro, the causative agent of African trypanosomiasis. *Science 202: 763-765.*

Hirumi, H., Doyle, J.J. & Cross, G.A.M. 1977. African trypanosomes: cultivation of animal-infective *Trypanosoma brucei* in vitro. *Science 196: 992-994.*

Hirumi, H. & Hirumi, K. 1989. Continuous cultivation of *Trypanosoma brucei* blood stream forms in a medium containing a low concentration of serum protein without feeder cell

layers. *J. Parasitol. 75: 985-989.*

Hirumi, H. & Hirumi, K. 1991 . In vitro cultivation of *Trypanosoma congolense* bloodstream forms in the absence of feeder cell layers. *Parasitology 102: 225-236.*

Hirumi, H., Martin, S., Hirumi, K., Inoue, N., Kanbara, H, Saito, A. & Suzuki, N. 1997. Cutlivation of bloodstream froms of *Trypanosoma brucei* and *T. evansi* in a serum-free medium. *Trop. Med. Intern. Hlth 2: 240-244.*

Ishii, T. & Bannai, S. 1985. The synergistic action of the copper chelator bathocuproine sulphonate and cysteine in enhancing growth of L1210 cells in vitro. *J. Cell Physiol. 125: 151-155.*

Kaminsky, R., Schmid, C. & Lun, Z.R. 1997. Susceptibility of dyskinetoplastic *Trypanosoma evansi* and *T. equiperdum* to isometamidium chloride. *Parasitol. Res. 83: 816-818.*

Kaminsky, R. & Zweygarth, E. 1989. Feeder layer-free in vitro assay for screening antitrypanosomal compounds against *Trypanosoma brucei brucei* and *T. b. evansi. Antimicrob. Agents Chemother. 33: 881 -885.*

Kaminsky, R., Zweygarth, E., Webster, P. & Chuma, F. 1988. Stability during in vttro cultivation and in vitro expression of drug resistance in trypanosomes. *ILRAD, Ann. Sci. Rep. p. 39.*

Le Page, R.W.F. 1967. Short term cultivation of *Trypanosoma brucei* in vitro at 37℃. *Nature 216: 1141-1142.*

Newton, B.A. & Le Page, R.W.F. 1967. Preferential inhibition of extranuclear deoxyribonucleic acid synthesis by the trypanocide Berenil. *Biochem. J. 105: 50.*

Olaho-Mukani, W., Mukunza, F., Kimani, J.K., Njoka, P.K. & Walubengo, J. 1993. Evaluation of the in vttro transformation technique to distinguish *Trypanosoma evansi* from cyclically transmitted *Trypanozoon* stocks. *Trop. Med. Parasitol. 44: 108-110.*

Ross, C.A. & Barns, A.M. 1996. Alteration to one of three adenosine transporters is associated with resistance to Cymelarsan in *Trypanosoma evansi. Parasitol. Res. 82: 183-188.*

Ross, C.A. & Taylor, A.M. 1994. Trypanocidal activity of a myristic acid analog in axenic cultures of *Trypanosoma evansi. Parasitol. Res. 80: 147-153.*

Shapiro, T.A. & Englund, P.T. 1990. Selective cleavage of kinetoplastic DNA minicircles promoted by antitrypanosomal drugs. *Proc. Natl. Acad. Sci USA 87: 950-954.*

Sutherland, D.V., Taylor, A.M. & Ross, C.A. 1993. A comparison of in vitro assay systems for the measurement of drug sensitivity of *Trypanosoma evansi. Trop. Med. Parasitol. 44: 208-212.*

Toohey, J.I. 1975. Sulfhydryl dependence in primary explant hematopoietic cells. Inhibition of growth in vitro with vitamin B12 compounds. *Proc. Natl. Acad. Sci. USA 72: 73-78.*

Trager, W. & Jensen, J.B. 1976. Human malaria parasites in continuous culture. *Science 193: 673-675.*

Yabu, Y., Takayanagi, T. & Sato, S. 1989. Long-term culture and cloning system for *Trypanosoma brucei* gambiense bloodstream forms in semi-defined medium in vitro. *Parasitol. Res. 76: 93-97.*

Zhang, Z.Q., Giroud, C. & Baltz, T. 1993. *Trypanosoma evansi* In vivo and in vitro determination of trypanocide resistance profiles. *Exp. Parasitol. 77:387-394.*

Zweygarth, E., Ahmed, J.S. & Rehbein, G. 1983. Cultivation of infective forms of *Trypanosoma (T.) brucei evansi* in a continuous culture system. *Z. Parasitenkd. 69:*

131-133.

Zweygarth, E., Gray, M.A. & Kaminsky, R. 199la. Axenic in vitro cultivation of *Trypanosoma vivax* trypomastigote forms. *Trop. Med. Parasit. 42: 45-48.*

Zweygarth, E. & Kaminsky, R. 1989. In vitro difflerentiation between *Trypanosoma brucei brucei* and *T. b. evansi. Trop. Med. Parasit. 40: 115-116.*

Zweygarth, E. & Kaminsky, R. 1990a. Direct in vitro isolation of *Trypanosoma brucei brucei* and *T. brucei evansi* from disease hosts with low parasitaemia. *Trop. Med. Parasit. 41: 56-58.*

Zweygarth, E. & Kaminsky, R. 1990b. Evaluation of an arsenical compound (RM110, mel Cy, Cymelarsan^{R}) against susceptible and drug-resistant *Trypanosoma brucei brucei* and *T. b. evansi. Trop. Med. Parasit. 41: 208-212.*

Zweygarth, E., Kaminsky, R. & Cheruiyot, J.K. 1989. A simple and rapid method to initiate *Trypanosoma (Trypanozoon) brucei brucei* and *T. (T.) brucei evansi* bloodstream form cultures. *Acta Trop. 46: 205-206.*

Zweygarth, E., Kaminsky, R. & P. Webster, P. 199lb. Dyskinetoplasia and loss of infectivity after long-term in vitro cultivation of a *Trypanosoma brucei evansi* stock. *Acta Trop. 48: 95-99.*

Zweygarth, E., Moloo, S.K., Kaminsky, R. & Gray, M.A. 1992. Axenic in vitro cultivation of Trypanosoma simiae bloodstream form trypomastigotes. *Acta Trop. 52: 79-81.*

Zweygarth, E.,Rehbein, G. & Ahmed, J.S. 1982. In vitro Kultivierung infektiöser Blutformen von *Trypanosoma (T.) brucei evansi* (Steel 1885). *Berl. Münch. Tierärztl. Wschr. 95: 407-408.*

Zweygarth, E. & Röttcher, D. 1986. In vitro cultivation of African stocks of *Trypanosoma (Trypanozoon) brucei evansi. Ann. Soc. belge Méd. trop. 66: 145-151.*