Comparative efficacy of different in vitro cultivation media for *Trypanosoma evansi* isolated from different mammalian hosts inhabiting different geographical areas of India

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Abstract The present study was undertaken to establish an optimal medium for primary culture initiation and maintenance of *T. evansi* isolated from different mammalian hosts of diverse geographical regions of India viz. donkey/1 (Hardoi, Uttar Pradesh), donkey/2 (Junagarh, Gujarat), pony/1 (Hisar, Haryana), camel/1 (Bikaner, Rajasthan) which represented isolates 1, 2, 3 and 4, respectively. Primary cultures were initiated with all four isolates in five different in vitro cultivation media with seeding density of 1×10^6 trypanosomes/ml. The parasites of all four isolates could remain viable only for 48 h in medium E (Alsever's solution) and for 72 h in medium A, C and D. Parasites reached to a maximum density ($2.5\text{--}3.75 \times 10^6$ /ml) within 24 h and thereafter, a sharp decline ($0.5\text{--}0.75 \times 10^6$ /ml) in the next 72 h was observed in 1, 2 and 3 isolates cultured in medium B. In isolate 4, parasite counts got more than doubled in 24 h and then decreased gradually up to sixth day post initiation of cultivation which thereafter increased gradually up to 34 days and a constant parasite number of 10^5 /ml could be achieved for 90 days in medium B. During this prolonged culture the trypanosomes retained their long slender morphology and infectivity to mice.

Keywords Culture media · In vitro cultivation · Livestock · *Trypanosoma evansi* isolates

Introduction

Trypanosomosis, caused by *Trypanosoma evansi*, is the most important haemoprotozoan disease infecting a wide range of

mammalian hosts causing high morbidity and mortality. The disease is a major constraint to livestock productivity on three continents viz. Asia, Africa and South America (Lucins 1988) and its significant socio-economic impact has been of increasing concern. The need for cultivation of trypanosomes in vitro is gaining much attention because of the advantage of allowing for the maintenance of parasites as an alternate of animal model. Further, axenic culture systems for the cultivation of bloodstream forms of *Trypanosoma* species may be helpful for study of drug efficacy, parasite biochemistry, immunology and molecular biology. The ability to grow the animal infective bloodstream forms of the parasite in the laboratory makes it possible to undertake research on the metabolic pathways of the organisms, the mechanisms of antigenic variation and other important problems such as the development of effective drugs for the disease control (Hirumi et al. 1977). Attempts with varying degree of success have been made for development of in vitro axenic culture system for *T. evansi* using different protocols over the last many years (Zweygarth et al. 1983; Baltz et al. 1985; Hirumi et al. 1997). But still no well suited protocol has been established that can be uniformly applicable to cultivate different isolates of the same parasitic form.

The present study was undertaken to establish an optimal medium condition for primary culture initiation and maintenance of *T. evansi* isolated from livestock species of different geographical areas of India.

Materials and methods

Parasite source and maintenance

Trypanosoma evansi stocks used in this study were isolated from different mammalian hosts of different geographical

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regions of India viz. donkey/1 (Hardoi, Uttar Pradesh), donkey/2 (Junagarh, Gujarat), pony/1 (Hisar, Haryana), camel/1 (Bikaner, Rajasthan) which represented isolates 1, 2, 3 and 4, respectively. These four different isolates were propagated in vivo in Swiss albino mice inoculated with laboratory cryopreserved trypanosomes. Initial inoculation in mice was done via intra-peritoneal route with freshly thawed 1×10^5 trypanosomes and post inoculation parasitaemia was monitored regularly.

Culture media for in vitro cultivation of *T. evansi*

To establish an optimal basic medium for primary culture initiation and maintenance, the trypanosomes of all four isolates were propagated in five different sets of media:

- Medium A Iscove's Modified Dulbecco's medium.
- Medium B Iscove's Modified Dulbecco's medium supplemented with 100 μ M bathocuproic acid (BCA), 1 mM sodium pyruvate, 100 μ M hypoxanthine, 16 μ M thymidine, 2 μ M 2-mercaptoethanol, 1.94 μ M L-cysteine, 60 μ M HEPES, 4 mM L-glutamine, 0.4 % BSA, 1 % antibiotics and 0.001 % holotransferrin, and 20 % FBS (HMI-9 medium modified slightly as described by Hirumi and Hirumi 1989).
- Medium C Minimum essential medium (MEM) supplemented with 1 mg/ml glucose, 2.2 mg/ml NaHCO_3 , 10 mM HEPES, 2 mM sodium pyruvate, 0.2 mM mercaptoethanol, 0.1 mM hypoxanthine and 20 % heat-inactivated horse serum (Kaminsky and Brun 1998).
- Medium D Phosphate buffer saline, pH 7.2 (136.89 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4 , 1.47 mM KH_2PO_4) supplemented with 1 % glucose.
- Medium E Alsever's solution (dextrose—20.5 g/l, sodium chloride—4.2 g/l, sodium citrate dihydrate—8.0 g/l, citric acid monohydrate 0.55 g/l).

Primary culture initiation

At peak parasitemia in experimental infected mouse with *T. evansi*, blood (100 μ l) was collected in a tuberculin syringe containing no anticoagulant from the heart of anaesthetised mouse and added immediately in culture medium under aseptic conditions. The blood trypanosomes were collected by centrifugation at $200 \times g$ for 5 min. Trypanosomes in supernatant were then used to initiate cultures in 1 ml culture medium in 24 well plates (Costar, Corning Inc., USA) at a seeding density of 1×10^6 trypanosomes/ml. The plates were incubated at 37 °C in a humidified environment of CO_2 incubator (5 % CO_2 , 95 %

air). The cultures were monitored every 24 h under phase contrast inverted microscope (Olympus) and parasite number was counted from each set of media for all the four isolates. The cultures were maintained by replacing 200 μ l fresh media in each well every 24 h. To check virulence of long term culture adapted parasites, the mice were inoculated (10^4 parasites/mouse) with 55, 75 and 85 days old in vitro cultivated trypanosomes.

Results

The observations on the five different culture media seeded with trypanosomes of four isolates were as follows:

- Medium A A sharp increase in the number of parasites (1.5–2.5 fold) was observed on day 1 in all the four isolates. But on day 2, the number decreased drastically and no motile trypanosome was observed on day 3 in all the four isolates.
- Medium B The count of parasites increased 2.0–3.75 times on day 1. On day 2, the number decreased significantly in all the isolates except isolate 1 in which a slight increase in count of trypanosomes was observed. Thereafter, the number of parasites declined continuously and on day 8, trypanosome count was reduced to nil in isolates 1, 3 and 4. The adaptation and long term survival of the parasites could only be observed in isolate 2. In this isolate, parasite number reached up to 10^4 /ml on day 6, started increasing thereafter and got doubled by day 8 (Table 1). Considerable increase in number of trypanosomes was observed from day 9 to 17 of cultivation. Thereafter, a rapid decline (3 times) in parasite population occurred by day 26 of cultivation. Second peak of increasing parasite population was observed from day 27 to 31 of cultivation (Fig. 1). Again the population declined rapidly up to 34th day of cultivation (Table 2). From day 35 onwards, a constant number of parasites (10^5 /ml) on alternate days could be harvested up to 90 days by replacing 200 μ l fresh medium every day (Fig. 1). The long term cultivated trypanosomes (55, 75 and 85 days), when inoculated, could develop fatal infections within 1 week in inoculated mice.
- Medium C On day 1, the parasite count decreased to 1/2 and 1/4 in isolates 3 and 4, respectively but remained constant in isolates 1 and 2. On day 2, the number further declined to zero in isolates

Table 1 Comparative efficacy of different media for in vitro cultivation of different *Trypanosoma evansi* isolates

Medium	Isolate	Isolate	No. of trypanosomes/ml ($N \times 10^4$)						
			Day 0	Day 1	Day 2	Day 3	Day 4	Day 6	Day 8
A	Donkey/1/Hardoi	1	100	250	8	0	0	0	0
	Donkey/2/Junagarh	2	100	150	1	0	0	0	0
	Pony/1/Hisar	3	100	200	5	0	0	0	0
	Camel/1/Bikaner	4	100	225	8	0	0	0	0
B	Donkey/1/Hardoi	1	100	375	400	10	1	0	0
	Donkey/2/Junagarh	2	100	200	6	2	2	1.0	2
	Pony/1/Hisar	3	100	250	50	10	6	1	0
	Camel/1/Bikaner	4	100	350	75	10	4	2	0
C	Donkey/1/Hardoi	1	100	100	0	0	0	0	0
	Donkey/2/Junagarh	2	100	100	1	0	0	0	0
	Pony/1/Hisar	3	100	50	1	0	0	0	0
	Camel/1/Bikaner	4	100	25	0	0	0	0	0
D	Donkey/1/Hardoi	1	100	200	5	0	0	0	0
	Donkey/2/Junagarh	2	100	75	0	0	0	0	0
	Pony/1/Hisar	3	100	50	5	0	0	0	0
	Camel/1/Bikaner	4	100	25	10	0	0	0	0
E	Donkey/1/Hardoi	1	100	150	0	0	0	0	0
	Donkey/2/Junagarh	2	100	50	0	0	0	0	0
	Pony/1/Hisar	3	100	40	0	0	0	0	0
	Camel/1/Bikaner	4	100	10	0	0	0	0	0

Fig. 1 Growth curve of *Trypanosoma evansi* (Donkey/2/Junagarh isolate) in medium B

1 and 4. On day 3, no motile trypanosome was observed in all the four isolates.

Medium D On day 1, the count of parasites decreased in all isolates except isolate 1 in which the number got doubled. On day 2, the parasite count declined further and no motile trypanosome was seen in isolate 2 and in remaining three isolates count was 0.5×10^5 – 1.0×10^5 /ml and died out by day 3 of cultivation.

Medium E In all four isolates, the parasite count decreased on day 1 and no motile trypanosome was seen on day 2.

Discussion

The in vitro axenic culture system that supports the growth of infective *T. evansi* is the need of the hour for studying biochemistry and immunology of the trypanosomes, the sensitivity of parasites to trypanocidal drugs, the mechanisms involved in drug resistance, the maintenance of parasites without using an animal model and to obtain large number of suitable organisms for use in the development of potential vaccines (Kaminsky and Zweygarth 1989; Hirumi et al. 1991, 1997). Although in vitro cultivation of developmental stages (amastigote and epimastigote forms) of

Table 2 Daily mean number of *Trypanosoma evansi* (Donkey/2/Junagarh isolate) in medium B

Day post initiation of in vitro cultivation	Parasite no. ($N \times 10^4$)	Day post initiation of in vitro cultivation	Parasites no. ($N \times 10^4$)	Day post initiation of in vitro cultivation	Parasite no. ($N \times 10^4$)
9	5	20	10	30	250
10	10	23	20	31	300
12	7.5	26	10	32	15
15	15	27	25	33	50
17	30	28	30	34	10
19	20	29	125	35–90	10

trypanosomes have been successfully developed but cultivation of blood stream forms (BSFs) still remained a challenge due to varying adaptive mechanism and nutritional requirement behaviour of different isolates of trypanosomes.

In the present study, the trypanosomes of all the four isolates survived for 48–72 h in culture media used in the study but multiplication behaviour was seen only up to 24 h in varying manner among different isolates. After 72 h the parasite remained alive only in medium B. Up to 72 h trypanosomes may remain alive in initial culture system without renewing the medium as reported by Zweygarth et al. (1983). These workers could not detect any motile trypanosome by day 3 when the culture medium was not renewed after initiation of in vitro culture of *T. brucei evansi* using fibroblast feeder layer in modified RPMI-1640 medium. This indicated that trypanosomes may survive initially for 3 days even if medium is not suitable for their adaptation and growth.

The study indicated that only medium B supported growth of trypanosomes in an in vitro system. The other four in vitro culture systems did not provide sufficient growth environment to the trypanosomes. Hirumi and Hirumi (1989) tested 18 different media for cultivation of *T. b. brucei* blood stream forms (BSFs). Among these media, HMI-9 medium was found best in terms of growth of these BSFs. In present study, in medium B three isolates (1, 3 and 4) could survive only up to 6 days except for isolate 2. The parasite number of isolate 2 also decreased gradually up to 6 days which thereafter increased gradually up to 34 days and thereafter a constant number of parasites ($10^5/\text{ml}$) could be harvested every other day for 90 days. This might be due to different nutritional requirement for adaptation and growth for three isolates other than isolate 2. Different isolates are known to exhibit different biological characteristics (Kundu et al. 2010). As such, they appear to require different conditions to initiate their growth in axenic culture system. These differences include origin of serum, presence of factors like pyruvate nucleotide precursors or macrophage feeder layer (Baltz et al. 1985). Carruthers and Cross (1992) studied growth of

bloodstream and insect form *T. brucei* on agarose plate using HMI-9 medium and reported that after a very short stationary phase trypanosomal viability began to decline rapidly and on 8th day viable trypanosomes fell below the level of detection. Although it is unclear what caused the rapid loss of viability, a critical nutrient in the medium may have been depleted.

During this prolonged culture the adapted trypanosomes retained their long slender morphology and infectivity even up to 85 days of cultivation, when inoculated in mice. Zweygarth et al. (1983) also reported no changes in either infectivity or pathogenicity of the trypanosomes cultured up to 140 days. Mice inoculated with cultured trypanosomes could develop fatal infections within 6 days. Baltz et al. (1985) also observed that in vitro cultured trypanosomes had all the characteristics of in vivo BSFs including morphology, infectivity, antigenic variation and glucose metabolism. Hirumi et al. (1997) observed that trypanosomes propagated in subcultures retained the morphological characteristics of long BSFs seen in the infected mouse blood and those harvested on day 71, and even day 84, gave rise to infection in all mice within 10 days after inoculation.

To develop a suitable protocol or optimal conditions for propagation of trypanosomes in axenic culture system further studies are needed on nutritional requirement during adaptation and growing phase, metabolic pathways and factors affecting the ability of *T. evansi* isolates to grow differently in a particular in vitro culture condition. However, the present protocol may be useful in efficacy study of trypanocidal agents against *T. evansi*.

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Ethical standard All authors certified that the animal experiments were carried out in the present work after approval of Institute Animal Ethic Committee and as per guidelines set by the Committee

for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Animal Welfare Division, Government of India.

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