Survival, activity and release of antigenic excretory secretory products and microfilariae of *Setaria digitata* maintained in artificial media

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Abstract The survival, activity and release of excretory secretory products from *Setaria digitata*, the filarial worm of cattle was studied. Adult female worms were maintained in vitro in DMEM and Tyrode solution. Worms incubated in DMEM were alive and very active for 2 days. The activity was moderate for another 2 days and after the fourth day increased mortality was observed. Antigenic excretory secretory products were also released. Worms incubated in Tyrode solution were very active without any mortality up to 4 h of incubation. Copious amount of ova and microfilaria were shed by the incubated worms in a time-dependent manner.

Keywords *Setaria digitata* · Cattle · DMEM · Tyrode solution · Excretory secretory products

Introduction

Among parasitic diseases, filariasis in man and livestock constitutes a major health problem in tropical countries. The debilitating effects and economic losses caused by these infections severely affect man and animal power resources in developing countries, most of which have agriculture based economics. Filariasis in man is a fearful and well documented vector borne parasitic disease affecting millions of people world wide since time

immemorial. On the contrary, relatively little progress has been achieved with respect to infections caused by filarial parasites of animals, particularly those affecting livestock. *Setaria digitata* is one of the common filarid worm of cattle. The adult worms are found in the peritoneal cavity and the larvae known as microfilariae are found in the blood. Immature forms of *S. digitata* are causing a condition called as “cerebrospinal nematodiasis or kumri” in unnatural hosts. The need for timely diagnosis and treatment of these filarial infections is inevitable and serodiagnostic approaches have to be standardized to detect carrier animals and those with low infection rates. Excretory secretory antigens play a major role in immunity to filarid nematodes of cattle and to extract these antigens worms need to be maintained in artificial culture media. This study was therefore undertaken to investigate the survival, activity and release of excretory secretory products from *S. digitata*, the filarial worm of cattle maintained in vitro in artificial media.

Materials and methods

The in vitro cultivation of adult *S. digitata* worms was done as per method of Mustafa et al. (1996) and Bal and Manoj (1995) with slight modifications. The in vitro cultivation in Tyrode solution was done as per the method of Robert and Steven (1976) and Dissanayake and Chandana (1988) with slight modifications.

Preparation of media

Dulbecco's Modified Eagle's Medium (DMEM), (Himedia, Mumbai) was the media used for cultivation of worms.

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The media was reconstituted in 950 ml of tissue culture grade distilled water at room temperature with constant stirring until the media was completely dissolved. The vial was again rinsed with tissue culture grade distilled water to remove all traces of the powder and 3.7 g of sodium bicarbonate was added. The contents were well dissolved and pH was adjusted to 7.4 and volume made up to 1,000 ml. The medium was filtered through a membrane filter (0.22 μ pore size) under sterile conditions. The medium was checked for sterility. Antibiotics—penicillin 1,000 mg/l, gentamicin 2 mg/l, cefatoxamine 20 mg/l and antifungal agent amphotericin B 2.5 μ g/ml prepared in sterile distilled water were added to the medium with sterile precautions.

Incubation of worms in DMEM

Adult female *S. digitata* worms ($n = 30$) were collected from the peritoneal cavity of freshly slaughtered cattle. Worms washed thoroughly in 3 changes of PBS every 15 min. The worms were then distributed in sterile glass dishes and milk dilution bottles containing 3 ml of medium per worm and incubated at 37 °C in 5 % carbon dioxide atmosphere. The medium was replenished every 24 h with fresh medium. The viability of the worms was monitored every day by observing for active motility. Mortality and other changes were also recorded every day. The spent media was collected everyday and was finally pooled. The membrane components were removed by centrifugation at 10,000 rpm for 60 min at 4 °C and the supernatant containing the antigen was dialysed against sucrose overnight under refrigeration followed by reverse dialysis overnight against PBS. The concentrated material was collected and used as excretory secretory antigen. The protein concentration was estimated by Bradford's method and the antigen was stored at –20 °C until further use. Antigenicity of the excretory secretory products released by the incubated worms was tested using counterimmunoelectrophoresis (CIEP) using known positive sera from microfilaraemic animals.

Incubation of worms in Tyrode solution

Thirty adult female *S. digitata* worms collected from the peritoneal cavity of freshly slaughtered cattle. Worms washed thoroughly in 3 changes of PBS every 15 min. The washed worms were then incubated in 100 ml of Tyrode solution at 37 °C for 4 h in an incubator with change of medium every hour. The worms were monitored for their activity. Mortality and other changes were also recorded. Shedding of microfilariae by the incubated worms was also

observed every hour. Total number of microfilariae released by the incubated worms every hour was also counted separately. The hourly collections were then pooled and centrifuged at 2,500 rpm for 10 min. The supernatant was discarded and the sediment containing the microfilariae was resuspended in five volumes of Tyrode solution. This was then sonicated 4 times at an interval of 2–3 s at 40 kHz, 1 mA for 90 s in a sonicator under ice. This sonicate was centrifuged at 10,000 rpm at 4 °C for 20 min in a refrigerated centrifuge. The supernatant was finally collected and used as antigen. The protein concentration was determined by Bradford's method and was found to be 5.3 mg/ml and the antigen was stored in aliquots at –20 °C until further use. Antigenicity of the microfilarial antigens released by the worms was tested using CIEP using known positive sera from microfilaraemic animals.

Results

All the worms incubated in DMEM were alive and very active for 2 days. The activity was moderate for another 2 days and after the fourth day mortality was more and they became sluggish and started disintegrating. Percent longevity on day 1, 2, 3, 4, 5, 6, 7 and 8 was 100, 100, 93.34, 86.67, 66.67, 46.67, 23.34 and 6.67, respectively. Worms were very active for the first 2 days, moderately active on day 3, 4 and 5, sluggish on day 6 and 7 and worms started disintegrating from day 8 onwards. Antigenic excretory secretory products were also released during incubation. The protein concentration was found to be 0.5 mg/ml.

Worms incubated in Tyrode solution were very active without any mortality up to 4 h of incubation. Copious amount of ova and microfilaria were shed by the incubated worms. The shedding of microfilaria increased in a time-dependent manner up to 3 h post incubation and then started declining. Copious amount of ova were also released by the incubated worms. Excretory secretory products were also released by the worms. The protein concentration was found to be 5 mg/ml.

Results of CIEP indicated that microfilarial antigen was superior to excretory secretory antigen in the diagnosis of Setarial microfilariosis in animals.

Discussion

Studies on in vitro maintenance of *S. digitata* worms in artificial culture media have been successfully carried out by several authors. Sugunan and Raj (1986) detected the origin of biologically active excretory secretory materials

from adult gravid female *S. digitata* worms during in vitro incubation at 37 °C in Tyrode solution for 4 h. Copious number of microfilariae and excretory secretory products were released by the worms, which showed a gradual increase up to 3 h and then declined. Hatching fluid emerging during the release of microfilariae was suggested as the major source of excretory secretory material. Decruse and Kaleysaraj (1988) observed excretory secretory material from different sites of reproductive tissue of healthy adult female *S. digitata* worms by maintaining them in Tyrode solution at 37 °C for 1 h. Robert and Steven (1976) conducted CIEP using microfilarial antigen and adult male worm antigen prepared from *Dirofilaria immitis* worms to detect filarial antibodies in dogs and cats infected with Dirofilariasis. They also prepared microfilarial antigen by immersing adult female *Dirofilaria immitis* worms in ice cold PBS at 4 °C for 1 h. In the current study, worms incubated in Tyrode solution were very active without any mortality up to 4 h with copious shedding of ova and microfilaria in a time-dependent manner. Dissanayake and Chandana (1988) obtained egg and microfilarial antigens of *S. digitata* by maintaining the adult female worms on Eagle's medium supplemented with antibiotics.

Sugunan and Raj (1990) reported the cross reactivity of *S. digitata* excretory secretory antigens with human filarial patients with Counterimmunoelectrophoresis. Mustafa et al. (1996) analysed the potential of excretory secretory antigens prepared by maintaining adult *Setaria cervi* in DMEM at 37 °C for the immunodiagnosis of human filariasis using CIEP. Bal and Manoj (1995) prepared

excretory secretory antigens by maintaining adult female *S. digitata* worms in DMEM supplemented with antibiotics at 37 °C under air with 5 % CO₂ with a change of medium every 24 h. In the present study, all the worms incubated in DMEM were alive and very active for 2 days with moderate for another 2 days and after the fourth day mortality was more and they became sluggish and started disintegrating. Antigenic excretory secretory products were also released during incubation.

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