PREPARATION OF EXCRETORY SECRETORY PROTEIN FROM BRUGIAN MICROFILARIAE ISOLATED FROM CANINE BLOOD

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Abstract

The article describes the culture of microfilaria of Brugia spp. for the extraction of excretory-secretory protein. The microfilariae were separated from the various components of canine blood using HiSep™ LSM by gradient centrifugation method. The microfilariae were cultured in RPMI-1640 media and incubated at 37°C. The spent media was collected at 12 hour intervals, filtered, pooled and stored at -50°C. Protein estimation was done by Bradford's method. The excretory-secretory protein produced can further be used in diagnostic tests.

Keywords: *Excretory secretory protein, Brugiasp. HiSep, Canine.*

Filariosis is an infection caused by nematodes of the superfamily *Filaroidea.* Canines are known to be infected with *Dirofilariaimmitis, D. repens, Brugiamalayi, B. pahangi, B. patei, B. ceylonensis, Acantho-chelionemadracunculoides, A. grassi* and *A. reconditum***.** As part of the Global Elimination of Lymphatic Filariosis Program, mass drug administrations are in place in endemic areas to control transmission. In canines, brugian filariosis is a common clinical diagnosis affecting almost 2% of the canine population(Ambily, 2009) and resulting in a wide range of manifestations including anorexia, conjunctivitis, lymphedema, scrotal edema. Kerala is endemic for human brugianfilariosis(Suma, 2013). However, the possibility of zoonotic transmission of brugian filariosis is not investigated completely. For complete evaluation of the clinical status, it is essential to increase the understanding of the physiology of the pathogen. Information available regarding brugian filariosis in animals is scarce. Hence the current study was undertaken to isolate the excretory secretory protein from the canine brugianmicrofilariae.

Materials and methods

Wet film examination from peripheral blood was carried out on canine patients presented to the University Veterinary Hospital, Kokkalai for various complaints

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and general check-up or vaccination. Blood smear examination was carried out for animals which were found to be positive for moving microfilariae more than 2 per field. On basis of Giemsa staining (Chirayath, 2015) and acid phosphatase staining (Chalifouxand Hunt, 1971) 15-30ml blood was collected from cases that were positive for brugian microfilariae, in EDTA. Blood was used for isolation of microfilaria.

Isolation of microfilaria

The collected blood was diluted with phosphate buffer saline (PBS) in the ratio of 1:1. Three milliliters of Hisep™LSM was placed in a 15 ml centrifuge tube (Beena, 2010). The diluted blood of a total volume of 12ml was layered over it. The tubes were placed in a fixed angle rotor centrifuge without brakes which was set at 400g for 30minutes at room temperature. The solution had separated into 4 layers. The top most layer contained plasma and thrombocytes. The next layer constituted of mononuclear cells and appeared hazy white. The third layer contained HiSep™LSM with microfilariae. The last layer had sedimented erythrocytes with a smaller percentage of microfilariae. All the layers were pippeted out individually. The layer containing HiSep™ LSM and microfilaria was suspended in PBS and centrifuged again at 400g for 5 minutes. The supernatant was discarded and the microfilarial pellet was resuspended in PBS and centrifuged with the above conditions. This wash was repeated with PBS once and a final wash with RPMI-1640 was done. A clear pellet of microfilaria free of erythrocytes was obtained.

Culture and extraction of protein

Following aseptic practices, the pellet was resuspended in RPMI-1640 media (containing 10mM HEPES buffer, 2mM L-glutamine, 4.5g glucose/l) and supplemented with 2g sodium bicarbonate. An antibiotic-antimycotic solution containing 100 µg/ml streptomycin, 100U/ml penicillin and 0.25 µg/ml amphotericin B was added to every ml of the media. The pH of the media was 7.2- 7.4. Eight milliliters of the solution was placed in a 100ml conical flask with a stopper in an incubator at 37 °C. The culture was examined every 12 hours for the viability of the microfilaria and live microfilaria was inoculated into fresh media. The spent media was collected every 12 hours by centrifugation of the media. The supernatant was filtered through a 0.22µm filter unit and stored at -50 °C. Spent media from several cultures were pooled and then filtered through Amicon® Ultra- 15 3kDa cutoff centrifugation units. The supernatant was stored at -50 °C. The protein concentration was determined by Bradford's assay.

Results & Discussion

The microfilariae pellet from 6 ml of undiluted blood gave an average of 25,000 microfilariae which was resuspended in 5-8ml of RPMI-1640. The microfilaria stayed alive for 26-36 hours at the conditions mentioned. Excretory secretory protein concentration was 435µg/ml as estimated by Bradford's method.

The filarids of the *Brugia spp*. reside in the lymphatics and the microfilariae circulate in blood. The most basic method for detection of the microfilariae is by examination of wet film from the peripheral blood. Staining of this further helps identify the species of microfilariae based on variations in the staining pattern. HiSep™ LSM is an iso-osmotic, low viscosity medium containing polysucrose and sodium diatrizoate, adjusted to a density of 1.0770± 0.0010 g/ml. It is routinely used for the isolation of mononuclear cells and lymphocytes from defibrinatedEDTA or heparin treated blood. Its composition is similar to that of Ficoll-Hypaque and both are available at various densities.

Rosewell Park Memorial Institute (RPMI) media are a series of media developed by Moore *et al.*(1997) for the culture of neoplastic cells in vitro. RPMI-1640 is used for a wide range of cell lines. It is the media of choice for the growth of microfilaria and is usually substituted with glucose, L- glutamine and sodium bicarbonate. Serum substitutes are not used since they interfere with the protein assays. Use of CO_2 incubators was associated with longer viability of microfilariae. Excretory secretory protein constitutes various proteins released from the parasite. Further studies of

these proteins may reveal new targets for drugs and prophylactics (Bennuru*et al*., 2009).

In this study ESP was extracted successfully. Further biochemical characterization will highlight their roles and may aid in the development of assays for mapping the extent of canine filariosis.

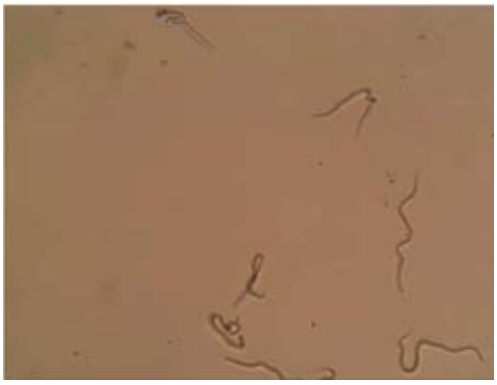

Figure 1. HiSep™ layer showing microfilaria (10X magnification)

References

Ambily, V.R. 2009.Clinico-therapeutic studies on canine microfilariosis*M.V.Scthesis*, Kerala Veterinary and Animal Sciences University, Pookode, Kerala, India.

Beena,V.2010. Anticancer and Nematocidal Properties of Crystal Proteins of *Bacillus thuringiensis*, *Ph.D thesis*, Kerala Veterinary and Animal Sciences University, Pookode, Kerala, India.

Bennuru, S., Semnani, R., Meng, Z.,Ribero, J.M., Veenstra, T.D. and Nutman, T.B. 2009.*Brugia malayi* Excreted/Secreted Proteins at the Host/Parasite Interface: Stage- and Gender- Specific Proteomic Profiling, *Plos Neglected Tropical Diseases,* **3**.

Chalifoux, L. and Ronald, D.H. 1971. Histochemical differentiation of *Dirofilariaimmitisand Dipetalonemareconditum*, *Journal of the American Veterinary Medical Association*, pp. 601-605.

Chenthamarakshan, V., Reddy, M., and Harinath, B. 1996.Diagnostic Potential of Fractionated *Brugiamalayi*Microfilarial excretory/secretory antigen for bancroftianfilariasis*Transactions of the Royal Society of Tropical Medicine and Hygiene,* **90**: 252-254.

Chirayath, D., Alex, P.C., and Pillai, U.N. 2015. Microscopic Characteristics of Canine Filariasis in Thrissur District of Kerala, *Indian Veterinary Journal*, **92**: 81 – 82.

Jones, T.C., Mott, K., and Pedrosa, L.C. 1975.A technique for isolating and concentrating microfilariae from peripheral blood by gradient centrifugation, *Transactions of the Royal Society of Tropical Medicine and Hygiene,* **69**:243-246.

Kwan-Lim, G.E., Gregory, W.F., Selkirk, M.E., Partono, F., and Maizels, R.M. 1989. Secreted antigens of filarial nematodes: a survey and characterization of in vitro excreted/secreted products of adult *Brugia malayi*, *Parasite Immunology,* **11**:629-654.

Moore, George, E., and Woods, L.K. 1977. Culture media for human cells-RPMI 1603, RPMI 1634, RPMI 1640 and GEM 1717. *Methods in Cell Science*.**3**: 503-509.

Suma, T.K. 2013.Indian Scenario of Elimination of Lymphatic Filariasis,*Medicine update. Kolkata: Association of Physicians of India*. pp. 6-9. ■