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Enhancement of viability of *Brugia pahangi* microfilariae using a Percoll gradient technique

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Abbreviations

DNA: deoxyribonucleic acid

EDTA: ethylenediaminetetraacetic acid

Abstract

Brugia pahangi is a common cause of animal lymphatic filariasis in cats, dogs and wild carnivores. It is used as a model for filarial infections because it is closely related to the human lymphatic parasite, *Brugia malayi*. To isolate microfilariae from blood, tools such as enzymes or phytochemicals have been used, but these methods could affect microfilarial viability. Hence, other techniques that cause the least effect on microfilariae are required. Percoll is a gradient medium used for cell preparation and is also used as a tool for the separation of microorganisms from blood. However, information on the separation of microfilariae from blood using Percoll gradients were last published some 30 years ago and the separation of the microfilariae of *B. pahangi* is little known. Therefore, the aim of this study was to evaluate the viability of the microfilariae of *B. pahangi* separated by Percoll gradient and simple centrifugation techniques. The use of Percoll gradient centrifugation for microfilarial purification showed a higher recovery rate of 90% compared to that from simple centrifugation. Parasite viability was assessed visually daily and the observations scored to estimate the survival rate. The reduction in motility of microfilariae separated by simple centrifugation was conspicuously reduced, while those separated using Percoll showed superior active motility until day 9. Percoll gradient centrifugation is a very viable alternativefor the isolation of microfilariae from blood, especially for the purpose of preparing microfilariae for in vitro study, since it requires the minimum centrifugation force, which is the least harmful to the parasite.

Keywords: *Brugia pahangi;*Isolation; Microfilaria; Percoll technique

Introduction

Brugia pahangi is a parasitic nematode belonging to the *genus Brugia* and is known as a common cause of animal lymphatic filariasis, a neglected tropical disease distributed worldwide [1]. Animals infected with *B. pahangi* are exposed to lymphadenopathy, lymphangitis and limb edema, which are similar in clinical appearance to those reported in humans. Recently, some reports have demonstrated that *B. pahangi* can cause clinical infection also in humans [2, 3]. Laboratory studies on the adults and microfilariae of B. pahangi have been established since 1960. In vitro cultivation is desirable for many reasons: to aid in the study of parasite biology or to evaluate the efficacy of antiparasitic drugs. Therefore, methods for isolation of the parasite from blood are required [4, 5]. In 1984, Percoll gradients were successfully used for the separation of microfilariae from blood. Percoll is a low-viscosity density gradient medium consisting of colloidal silica coated with polyvinylpyrrolidone (PVP) and is known as a tool for the preparation of cells and cellular particles and is also used for the separation of microorganisms from whole blood [6]. All types of cells present in blood, including erythrocytes, lymphocytes, monocytes, neutrophils, eosinophils, basophils and natural killer cells, can be fractionated by using various gradients of Percoll [7-13]. In addition, most of other cell types, such as liver cells, bone marrow cells, Leydig cells and spermatozoa can be purified and enriched by using Percoll [14-18]. In the parasitology field, Percoll is used to purify many types of microorganisms infecting either intracellularly or extracellularly, including Babesia, T*heileria, Plasmodium* and the microfilaria of filarial nematodes [19-23]. Even in fecal cyst loads in stool samples, Percoll can be used for separation and provides a good yield of *Entamoeba histolytica* [22].From three decades ago, the studies of microfilarial isolation method were published. Accordingly, current information about the isolation of microfilariae from blood on Percoll gradients needs to be given. This study was performed to validate the separation of the microfilariae of *B. pahangi* on Percoll gradient medium and observed the survival rate of the microfilariae in *in vitro* cultivation.

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Materials and Methods

Blood samples

Five milliliters blood samples taken from dogs naturallyinfected with *Brugia* were obtained from the Veterinary Teaching Hospital of Mahidol University. Samples were first screened by using the traditional Giemsa staining technique before undergoing molecular identification and parasite isolation. The animal use protocols of Chulalongkorn University Animal Care and Use Committee, Chulalongkorn University (Animal Use Protocol No. 1931004) were followed.

Parasite identification

Brugia spp. infections were examined in blood smears with Giemsa staining for morphological identification. Species identification and PCR using primers 5´-AGTGCGAATTGCAGACGCATTGAG-3´and 5´-AGCGGGTAATCACGACTGAGTTGA-3´ were performed [24]. The PCR procedures were performed according to Rishniw et al. (2006). The PCR products were run on a 2% agarose gel and visualized under UV illumination [26].

Microfilariae preparation

Microfilariae were counted and total microfilaremia calculated. Total blood samples were divided equally for two separation methods: simple centrifugation (Group1) and Percoll in 0.25 M sucrose gradient (Group 2).

Simple centrifugation method

EDTA–blood was spun at 8500 rcf for 5 min at room temperature. Plasma above the buffy coat layer was placed into individual tubes and 20 µl of microfilarial content from the plasma layer taken and quantitated. The total content was washed three times with RPMI-1640 medium.

Percoll gradient method

The procedure for using Percoll gradient medium to separate microfilariae from whole blood has been described previously [23]. Briefly, the EDTA–blood was diluted with RPMI-1640 medium in the ratio of 1:1. Iso-osmotic Percoll (IOP) was prepared by mixing nine parts of Percoll with one part of 2.5 M sucrose. Dilutions of the IOP in 0.25 M sucrose were made to obtain 35 and 40% media of density 1.068 and 1.074, respectively (Figure 1). For the isolation of microfilariae, 2 mL of 40% IOP medium was placed in a 15-mL conical centrifuge tube and overlayed by the same volume of 35% IOP medium. Then, 0.5 mL of diluted blood was added to the gradients. Tubes were spun at 2000 rcf for 30 min at room temperature. After centrifugation each fraction of plasma and motile microfilariae layers were transferred to individual tubes, from which 20 µL of microfilarial content from this layer was taken and quantitated. The total volume of microfilarial layer was washed three times with RPMI-1640.

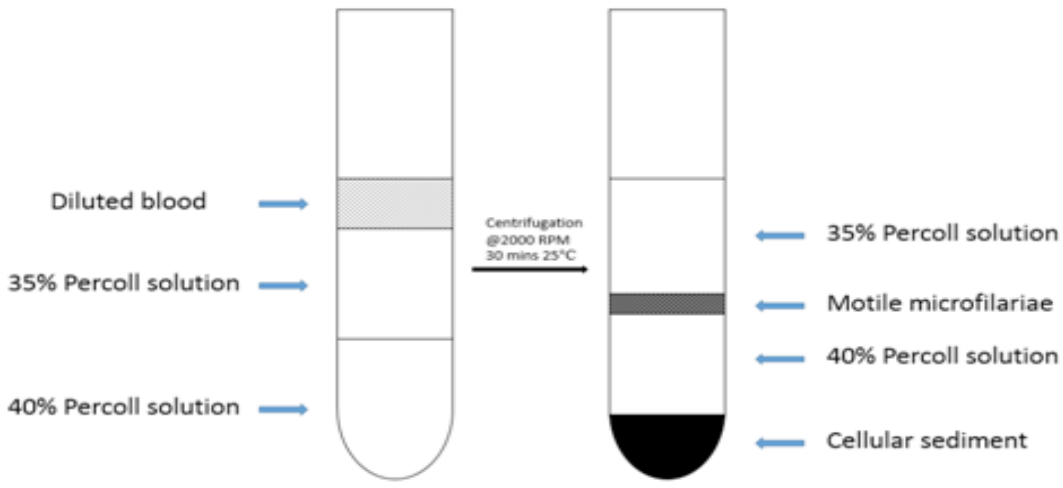

Figure 1: Percoll centrifugation resulted in separation of the microfilariae as a separated band in between the 35% and 40% plasma with Percoll layers

Parasite viability assay

Microfilariae recovered from the two methods were each transferred to 96-well plates, adding RPMI-1640 supplemented with 25 mM HEPES buffer, 2 mM glutamine, 10% fetal calf serum, streptomycin, amphotericin-B and penicillin at 37 °C in a 95% air–5% CO_2 atmosphere. Microfilarial motility and death were assessed visually by inverted microscope (Olympus, USA). The observations were conducted daily for nine days and scored as 0–4 as described previously by Rao and Weil (2002) [25]. The data were analyzed by Mann–Whitney U test and differences were considered significant with P values of <0.05.

Results

Identification of parasite

Sheathed microfilariae of *Brugia spp* were morphological confirmation by Giemsa staining technique. Molecular species confirmation was then conducted byusing primers specific for the *5.8 S-ITS2-28 S* gene of *B. pahangi* amplified DNA fragments of the anticipated product sizes of 665 bp in all samples.

Recovery rate

In Group 1, after centrifuging at 8500 rcf, three layers: plasma layer, buffy coat layer and packed red blood cell layer, were seen. The plasma layer that interfaced with the buffy coat layer was taken separately. Group 2, after centrifuging with Percoll, consisted of four layers: plasma with 35% and 40% Percoll layers, motile microfilariae layer and cellular sediment, were seen in Figure1. From each group, 20 µL was placed onto a microscopic slide and the microfilariae counted and the numbers of microfilariae were calculated. The percentage recoveries of the parasite from each group are presented. Approximately 90% of microfilariae were distributed in the motile microfilariae layer of Group 2, whereas in Group 1 the calculated recovery rate was only 43%.

Motility score

Microfilariae from the two separation methods were cultured with complete RPMI culture media. Motility was assessed visually and the observations were scored as 0–4 to estimate the survival rate. The scoring of motility was compared between the two groups. In Group 1, microfilariae separated by simple centrifugation method, the motility scores were reduced markedly and reached score 0 on day 4, while microfilariae in Group 2, separated by using Percoll, showed the highest active motility score of 4 until day 9 (Fig. 2). Comparing the survival rate between two groups, they was significantly different (P < 0.05)

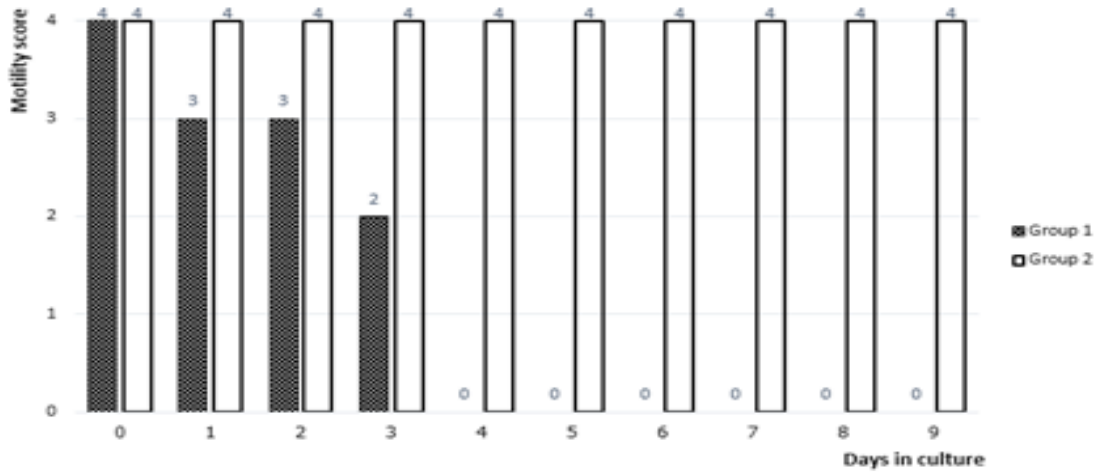

Figure 2: Microfilarial motility was observed daily. Results showed the reduction in motility compared between the two groups; microfilariae in Group 1 were separated by simple centrifugation while those in Group 2 were separated by Percoll gradient.

Discussion

Since 1977, Percoll has become the density gradient medium of choice for many researchers worldwide for use in separating cells and other microorganisms [8–23]. This study was conducted to evaluate the effectiveness of Percoll as a tool for microfilarial isolation from infected blood. Results showed that separation with Percoll (Group 2) provided a better recovery rate of microfilariae (90%) compared to simple centrifugation. This result is similar to those of Chandrashekar et al. (1984), who achieved percentage recoveries of *B. malayi, B. pahangi* and *Dipetalonema viteae* microfilariae by Percoll gradient separation of 85–97%. Nevertheless, some laboratory techniques have been described for microfilarial purification, such as the use of saponin, phytohemagglutinin, enzymes or distilled water, which could affect the viability of microfilariae [27-30]. For the microfilariae of *W. bancrofti,* Jones et al. (1975) described a method for the separation of microfilariae from infected blood using Ficoll-Hypaque gradient, but the isolation of the parasite from blood cells was not successful in that the yield was low and

the microfilariae remained contaminated with white blood cells [31].

Parasite viability was assessed by motility observation and scoring. Results revealed that Group 2 retained their motility, showing the highest activity score until day 9, whereas the motility score in Group 1 decreased sharply from the first day of the experiment and reached a score of 0 within four days. Considering the use of Percoll for other parasites, a 65% Percoll concentration was found to be optimal for separation of *Babesia bovis* merozoites (i.e., mature exoerythrocytic stage) and a 100% Percoll stock medium was optimal for enrichment of infected erythrocytes. For *Theileria*, a structure called "veil" could be purified from infected bovine erythrocytes by using Percoll. In studies of *Plasmodium*, Percoll was used for the separation of host erythrocyte membrane from malarial parasites. The recovery of the erythrocyte membranes was 65–70%, whereas parasite recovery was 80–90%, and the relative purity was 85–90%. Moreover, these parasites that were isolated by Percoll gradient centrifugation retained their morphology intact and were successful for *in vitro* cultivation [19-22]. It is evident that microfilariae purified by Percoll gradient separation are more viable than those purified by simple centrifugation. There are several possible explanations: i) Percoll has low viscosity, allowing more rapid sedimentation and the use of lower centrifugal forces, which is less damaging for the microfilariae; isolating microfilariae by simple centrifugation requires higher centrifugal force [32, 33]; ii) When using Percoll medium the microfilariae were free of cellular components, especially white blood cells, which can affect their viability [34]. Other advantagesof the use of Percoll gradient medium are that it is currently the most rapid method available for preparing viable microfilariae, it uses isotonic conditions throughout, it does not require resuspension steps to isolate the microfilariae and it does not require an ultracentrifuge [35].

Conclusions

Percoll gradient medium is a very viable alternativefor the separation of microfilariae, especially for the purpose of preparing them for *in vitro* study. In this study, this technique provided up to 90% recovery rate and microfilarial viability showed the highest score until the end of the study.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Ethics approval and consent for publication

Not applicable

Consent for publication

Not applicable

Consent for publication

The authors declare that they have no competing interests.

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