Chapter 5

5.1. Introduction

Diagnosis is an essential element in the management of disease, both at the level of individual patient care as well as at the level of disease control especially in populations living in endemic areas. But the currently used diagnostic methods have their own limitations. The most widely used method for diagnosis of filarial infection is microscopic examination of patient blood for the presence of microfilaria (mf) which has limited sensitivity and is not suitable for large-scale screening of human populations in endemic areas (Walther et al., 2003). This method is tedious and not convenient both for the patient as well as the investigator due to the nocturnal periodicity of mf. In epidemiological surveys, the alternate technique used to detect and characterize filarial parasites in vectors includes the collection, dissection and microscopy, this technique is not reliable because the mosquitoes may carry both human and animal filarial parasites, which cannot be distinguished biochemically or morphologically (Sim et al., 1986a: Sim et al., 1986b). Currently there is no fast, reliable and sensitive biochemical or immunological method for distinguishing closely related species or sub-species of filarial parasites. In order to overcome some of these shortcomings, improved methods for diagnosis of filarial infections are needed to study the transmission, epidemiology and facilitate surveillance activities to monitor control efforts (Chandrashekar et al., 1994).

A more perfect, rapid format, immunochromatography card test (ICT) which detects circulating antigen is available for bancroftian filariasis (McCarthy et al., 2012 and WHO 2005) but not for other filarial infections. Detection of microfilariae in
conjunction with antibody testing, mainly in clinical settings, is being used as an interim measure for brugian filariasis (McCarthy et al., 2012; Weil et al., 2007). However, the antibody tests indicate and later exposure rather than the active infection (Rao et al., 2007; Vincent et al., 2007) and do not distinguish between bancroftian and brugian filariasis (Lammie et al., 2004), thereby limiting their use for surveillance in areas where these infections are co-endemic. Antigen and antibody based techniques (ELISA and western blotting) are highly sensitive and specific, used to diagnose the disease but are losing the preference over the molecular level methods. These methods require highly purified antigens as well as raising of antibodies against the antigen. This whole process makes it time consuming as well as need highly skilled working hands with no room for error. These processes can also be outsourced to companies, which also makes it an expensive process.

In recent years, studies have been focused on the development of specific diagnostic methods based on deoxyribonucleic acid (DNA) probes to replace laborious traditional method for diagnosis of disease (Chanteau et al., 1994). The molecular biological techniques such as DNA probe development (Siridewa et al., 1994; McReynolds et al., 1986; Poole et al., 1990; Williams et al., 1988) and repeated DNA polymerase chain reaction (PCR) method (Lizotte et al., 1994; McCarthy et al., 1996; Zhong et al., 1996) had been used in W. bancrofti or B. malayi detection and have provided the opportunity for improved diagnosis. The polymerase chain reaction (PCR) is recently being used to enhance the sensitivity of DNA based parasite detection assays in mosquitoes and in human blood samples by detecting the filarial DNA. These test encountered some problems as mosquitoes contain PCR inhibitor (Dissanayake et al.,
1991) due to which DNA undergoes degradation during collection, transport and storage (Cooper et al., 1998) although the PCR assay was found to be specific but sensitivity was considerably single in samples with low microfilarial counts (Lizotte et al., 1994; Farid et al., 2001). In search of a promising diagnostic tool few genes were identified to be conserved in the human and bovine filariasis. In the present study the mitochondrial cytochrome c oxidase subunit 1 (cox1) gene of *S. cervi* and *B. malai* and highly repeated sequences of Hha1 gene of *B. malayi* were studied.

5.2. Materials and Methods

5.2.1. PCR Amplification for Hha1 gene *B. malayi* by Using EST Primer

The primers were designed for filarial adult parasite specific DNA based on sequence from DNA databank, EST (Gene Runner), highly repeated sequence of filarial DNA and homologous sequence in the genome of *B. malayi*. These primers were purchased commercially (Imperial Life Science, USA). The forward and reverse primer for *B. malayi* were designed (Hha1 F 5’-GCG CAT AAA TTC ATC AGC-3’, Hha1 R 5’-GCG CAA AAC TTA ATT ACA AAA GC-3’). The PCR kit (Fermentas Pvt. Ltd, USA) was used and the temperature conditions for thermal cycler (BioRad, USA) were of 35 cycles at 94° C for 5 min followed by 94° C for 1 min, then 59.6° C for 1 min and 72° C for 1 min and finally at 72° C for 10 min. The above designed primers were used for amplification of the DNA from *B. malayi*. The amplified gene product size was checked on agarose gel (1.5%) electrophoresis.
5.2.2. PCR Amplification of Cox 1 Gene of *S. cervi*

The volume of DNA sample was made up to 100 µl with the DNA binding buffer (100 mM Tris-HCl (pH 7.5), 100 mM NaCl). The primers were used to amplify the filarial genes of *S. cervi*. The amplification reactions were performed using the primers. The PCR was performed using the same thermocycler conditions as used above in the case of *B. malayi* except the annealing temperature was set at 55°C. PCR products obtained were run on the 1.5% agarose gel, visualized under U.V. at 265 nm in a transilluminator (Bangalore Genie, India).

5.3. Results and Discussions

5.3.1. PCR Amplification for HhaI Gene of *B. malayi*

The microscopic examination of *B. malayi* microfilariae and adult were done before the isolation of DNA. This study revealed the typical morphology of the *B. malayi* microfilariae and adult parasite as shown in Fig. 3.6, 3.7. The *S. cervi* mf and adult parasite as shown in (3.8 A & B). The parasitic DNA isolates were isolated and PCR amplified using the primer (Hha1), specific to *B. malayi* in a thermal cycler (Bio Rad, USA). The specific primer used for detecting the parasites was found to give optimum yield of the amplified fragment size of 322 bp for *B. malayi* (Fig.5. 1). Similarly, the tandemly repeated 320-bp HhaI sequence of *Brugia* has been used as a target for PCR assays by many researchers (Lizotte *et al.*, 1994; Williams *et al.*, 1988; Mishra *et al.*, 2005; Rao *et al.*, 2002). Vasuki *et al.* (2001) developed a rapid and simplified method for the detection of *B. malayi* using HhaI forward and reverse PCR primers and further, *B.*
malayi 322 bp specific band was found as the PCR amplified products when electrophoresed on a 2% agarose gel. Further, Mishra et al. (2005) also used HhaI and SspI primers for the combined detection of B. malayi and W. bancrofti, respectively using single PCR.

**Figure 5.1:** PCR amplified product of HhaI gene B. malayi (Marker Size range-100bp-5kb Fermentas, USA)

### 5.3.2. PCR Amplification for Cox I Gene by Using the Primer

PCR amplification for Cox I gene was carried out by using Cox I gene specific primer and a size 680 bp of fragment was amplified. The S. cervi Cox 1 fragment of 680 bp in length was confirmed on 1% agarose gel (Figure. 5.2) when visualized at 265 nm under UV in a transilluminator (Bangalore Genie). In a concurrent study, Alasaad et al. (2012) used sequences of mitochondrial cytochrome c oxidase subunit 1 (cox1) gene for the phylogenetic analysis of S. cervi.
The diagnosis of lymphatic filariasis is presently based on microscopic examination of night blood, and morphological assessment of stained microfilariae. This approach has limited sensitivity and is not suitable for disease-endemic areas, where large scale screening has to be done and investigation is unable to distinguish among the human filarial parasitic species and animal filarial parasites due to their similar morphology. Use of molecular-based diagnostic tools is considered more accurate since they detect active infection and have been used in laboratories for reliable differential identification of filarial parasites. Several polymerase chain reaction (PCR) based methods have been used to amplify DNA in blood from *B. malayi* and *B. timori* (Lizotte *et al.*, 1994; Rahmah *et al.*, 1998; Rao *et al.*, 2006) and *W. bancrofti* (Chansiri *et al.*, 2002; Fink *et al.*, 2011; Rao *et al.*, 2006). Molecular monitoring of insect vectors by PCR is also the preferred method for xeno-diagnosis and has been used extensively for *W.bancrofti* (Ramzy *et al.*, 1997; Rao *et al.*, 2006; Intapan *et al.*, 2009) and to a lesser extent for *B. malayi* (Laney *et al.*, 2008)
The sensitivity and specificity of this assay has demonstrated its potentiality to be used as an efficient tool for diagnosis of filariasis in blood samples. Prevalence of the disease can be estimated from the infection status of the mosquitoes and the human population in an area. Particularly, in endemic areas, the advantage of this method could be adopted in screening large collections of mosquitoes for parasite detection (Vasuki et al., 2001). Apart from the factors like specificity and sensitivity, cost effectiveness is the essential component of any technique that is employed for diagnosis. This method takes care of the cost factor by diagnosing either parasite in a single step PCR. The most positive application it offers is to screen the status of endemicity in an area by handling large number of blood samples and mosquitoes in relatively short time and at low cost. The multiple applicability of this method proves its effectiveness to be used as a diagnostic tool for determination of filariasis.

The parasitic DNA was isolated from the adult parasites (B. malayi and S. cervi) and Hha1 gene of B. malayi was amplified using the Hha 1 gene specific primers in a thermal cycler (BioRad, USA). The specific primer used for detecting the parasites was found to give optimum yield of amplified fragment size of 322 bp for B. malayi (Fig. 5.1). The recognition site for Hha1 in B. malayi repeats contains the Alu I and Rsa I recognition sites. Thus it could be concluded that the Hha1 primer can be used to detect the B. malayi DNA specifically in the patient samples.

The Brugia Hha I repeat was selected as the biomarker for PCR based diagnostic test for brugian filariasis. The repeats are non-protein coding, approximately 322 bp in length, and arranged in direct tandem arrays. They comprise between 1–12% of the B
*.malayi* genome (Poole *et al.*, 2012) and are highly conserved, with the nucleotide identity of the repeats used in this study varying from 82–98%. In order to target the greatest number of repeats and maximize assay sensitivity, primers were designed based on the most highly conserved nucleotide blocks in a consensus sequence (Chen *et al.*, 2002).

The bovine filarial parasitic DNA was isolated from the adult parasites (*S. cervi*) and Cox1 gene of *S. cervi* was amplified using the Cox1 gene specific primers in a thermal cycler (BioRad, USA). The specific primer used for detecting the parasites was found to give optimum yield of amplified fragment size of 680 bp for *S. cervi* (Fig. 5.2). Cox 1 gene is conserved in filarial parasites both human and bovine therefore appears to be a potential drug target.