[BIO40] Homologs of the *Brugia malayi* diagnostic antigen *Bm*R1 are present in other filarial parasites, but induce different humoral immune response

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Introduction

Lymphatic filariasis, caused by *Brugia malayi* and *Brugia timori*, is endemic in several Asian countries and infects ~ 13 million people. The WHO initiated Global Program for Elimination of Lymphatic Filariasis (GPELF) aims to eliminate the disease as a public health problem by the year 2020. To ensure its success, a sensitive and specific field applicable diagnostic tool is needed for mapping and monitoring all phases of the program. For bancroftian filariasis caused by *Wuchereria bancrofti*, the ICT antigen card test is widely used for this purpose. This test is based on the detection of a circulating adult worm antigen of *W. bancrofti* (Chandrashekar *et al.,* 1999). Detection of anti-filarial IgG4 antibody provides a good alternative diagnostic tool for brugian filariasis as this antibody subclass has been shown to be elevated in active infection and declines post-treatment (Haarbrink *et al.,* 1999). Recombinant antigen-based antibody assays would be preferable over assays based on parasite extracts since the former allow for unlimited supply of well-defined antigens.

The *Bm*R1 recombinant antigen, expressed by gene pPROEX/ *Bm17DIII* (GenBank accession no. AF225296) has been shown by us to be a highly specific and sensitive antigen for IgG4 assays to detect exposure to both *B. malayi* and *B. timori* infections. The antigen was used in ELISA and rapid test (Brugia Rapid) formats and evaluation in various laboratories and field trials revealed a sensitivity of 93%-100% in detecting mf positive individuals (Supali *et al.,* 2004; Rahmah *et al*., 2003b).

The *Bm*R1 antigen was very specific (99% -100%) with respect to reactivity with sera from non-filarial infections (Rahmah *et al*., 2003b). The highest prevalence of cross-reacting antibodies in other filarial infections was found in *W. bancrofti*, followed by *Loa loa*; while only one sample (of 9) patients with *Dirofilaria* infection. However cross-reactivity was not found in patients with *Onchocerca volvulus* or *Mansonella* infection (Fischer *et al.,* in press, Rahmah *et al.,* 2003b).

Due to its diagnostic significance, it is therefore important to characterize the *BmR1* antigen more closely. The varying degree of *Bm*R1 recognition in other filarial infection raises the question whether the homologous antigen is also present in *W. bancrofti*, *L. loa* and *O. volvulus*.

Materials and methods

cDNA and genomic DNA

W. bancrofti microfilaria (mf), adult male and adult female cDNA libraries were obtained from Filarial Genome Project Resource Centre (Smith College, Northampton, USA). Genomic DNA of *W. bancrofti* was prepared from mf provided by the author from ICMR; comprised two negative samples and two positive samples by Brugia Rapid test. *L. loa* L3 and *O. volvulus* mf cDNA libraries were kindly provided by Dr. Peter Fischer from Germany.

PCR

PCR primers used to amplify the entire *Bm17DIII* gene sequence, were RNF (24 mer) 5'att act gat tag tat ttt atc gtt 3' and RNR (24 mer) 5'atg ata aaa atg aat gag aaa tat 3'. λ phage plaques were amplified and the DNA was extracted using λ DNA extraction kit (Qiagen, Germany). PCR was then performed in a thermocycler (Perkin Elmer, USA) at: 94ºC, 5mins; 55ºC, 5mins; 35 cycles for 94ºC, 45sec; 55ºC, 45sec & 72ºC, 90sec; 72ºC, 10mins. *W. bancrofti* mf genomic DNA from was prepared using Genispin Tissue DNA Kit (BioSynTech, Malaysia) and amplifications was performed at: 94ºC, 5mins; 35 cycles for 94ºC, 1min; 55ºC, 1min & 72ºC, 1min; 72ºC, 10mins.

TOPO cloning and DNA sequence analysis

PCR products of the homologs gene were cloned into TOPO-TA vector (Invitrogen, USA), and then transformed into *E. coli* TOP10 host (Invitrogen). The recombinant plasmids were amplified, purified using QIAprep® Spin Miniprep Kit (Qiagen, Germany), and sequenced (ACGT Inc, USA) before analyzed using vector NTI software.

Subcloning, expression and purification of Ov17DIII/Loa17DIII

The *Bm*17DIII gene homolog in *O. volvulus/L. loa* were subcloned into a bacterial expression vector, pPROEX-HT which contain 6-His tag (Life Technologies, USA), and then transformed into *E. coli* TOP 10 host cells.

The recombinant bacteria were cultured in Terrific broth and placed in a 37°C shaker incubator until the OD_{600} reached 0.5. The culture then induced with 1 mM IPTG for 3 hrs at 30°C. The bacterial pellet was reconstituted with lysis buffer containing 50 mM Tris HCl (pH 8.5), 5 mM 2-mercaptoethanol and a cocktail of protease inhibitors (Roche Diagnostics, Germany). The suspension was sonicated, followed by centrifugation at 12000xg, 30 minutes; and the resulting supernatant purified using Ni-NTA resin (Qiagen) and buffers containing imidazole.

ELISA

The methodology employed was as previously reported (Rahmah *et al*., 2001a). Briefly, microtiter wells (Nunc, USA) were coated with 100 µl either with *Bm*R1 (20 µg/ml) or *Ov-Bm*R1/*Loa-Bm*R1 recombinant antigens (5, 10 or 20 µg/ml) in $NaHCO_3$ buffer (pH 9.6). After a blocking step, serum samples (1:20 or 1:50 or 1:100) were incubated for 2h, followed by 0.5 h incubation with the secondary antibody HRP conjugated to monoclonal anti-human IgG1 (1:6000), IgG2 (1:1000, 1:2000), IgG3 (1:1000, 1:2000) or IgG4 (1:4500) (CLB, Netherlands). Subsequently, ABTS substrate (Roche Diagnostics) was added for 30 minutes before the optical densities (OD) were read at 410 nm with an ELISA spectrophotometer (Dynatech, USA).

A panel of 262 sera samples was from pre-existing serum banks. The *O. volvulus* sera were from mf positive individuals from Western Uganda. *L. loa* sera were from mf positive individuals from the clinical department of BNI. *W. bancrofti* sera were from India; while sera from *B. malayi* infections, endemic normals (both disease and Brugia Rapid negative), non-endemic normals (healthy blood donors) and other parasitic infections were from Malaysia. Infections with other parasites comprised patients from Malaysia whose stool specimens were positive for parasite ova/larva (single or mixed infections with Ascaris *lumbricoides, Trichuris trichiura*, hookworm, *Strongyloides stercoralis*); patients with clinical presentation and serology consistent with toxocariasis and amoebiasis; and a patient with *Gnathostoma spinigerum* isolated from the eye.

Results

Identification of the BmR1 homolog

PCR of *W. bancrofti* cDNA libraries and *W. bancrofti* genomic DNA (from all 4 mf samples) produced a single band of 618 bp. A total of 12 recombinant clones, from six TOPO reactions (2 from mf cDNA, 1 from adult female cDNA, 1 from adult male cDNA and 2 from mf genomic DNA) were sequenced. A total of 31 DNA sequencing reactions were analyzed and all obtained sequences were identical. Comparison of the obtained nucleotide sequence showed that it is 100% identical to the cDNA sequence of *Bm*R1, irrespective whether the template DNA came from cDNA libraries, or mf originated from individuals positive or negative for Brugia Rapid test.

For identification of the cDNA of the *Bm*R1 homolog in *O. volvulus* and *L. loa*, a total of 5 and 3 recombinant clones were sequenced respectively (comprising a total of 20 reactions). The *O. volvulus* and the *L. loa* homolog were 100% identical to each other; and only two bases were different from the *B. malayi* and *W. bancrofti* homolog, *i.e* at 97bp and 483bp, and only one amino acid difference observed. The uncharged, polar isoleucine at position 33 was substituted by a neutral threonine. (Figure 1)

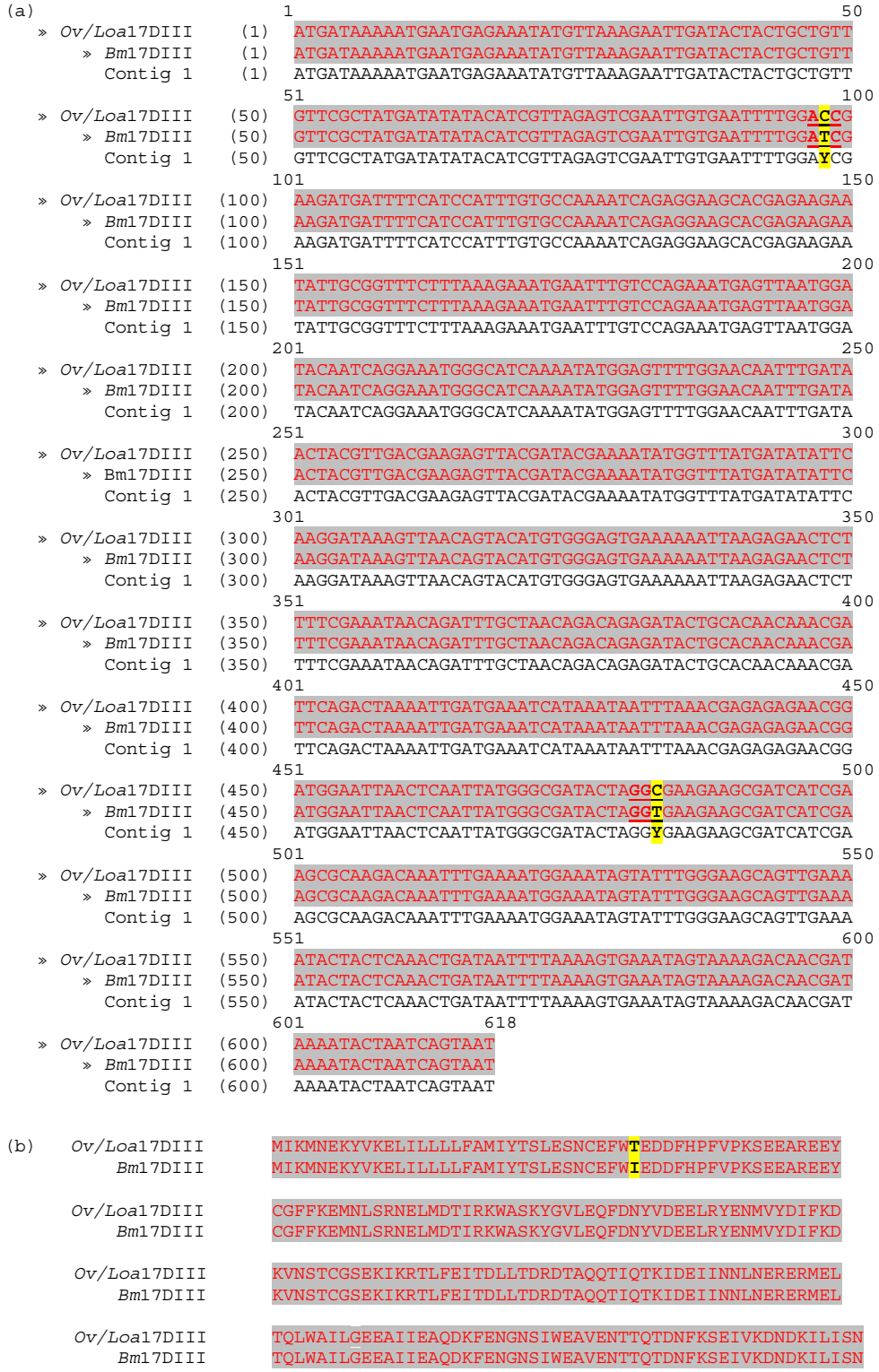

FIGURE 1 Data showed the DNA and amino acid sequence of *Bm17DIII* and its homologs in *W. bancrofti*, *O. volvulus* and *L. loa,* (a) *Bm17DIII* and its DNA homologs with two bases difference i.e. at 98 & 483; (b) *Bm*R1 amino acid sequence was identical with *W. bancrofti*; whereas with *O. volvulus* and *L. loa*, a difference occurred only at one amino acid coded by bases 97-99 i.e. a change from Ile (ATC) to Thr (ACC).

Antibody reactivity to the B. malayi BmR1 antigen and its homolog

For IgG4-ELISA, serum samples that demonstrated average optical density (OD) readings of ≥0.300 were considered to be positive (*Rahmah et al.,* 2001a). The IgG4-ELISA results showed that both recombinant antigens were 100% identical in reactivity with the various categories of sera (Table 1).

TABLE 1 Comparison between IgG4 reactivities of *Bm*R1 and *Ov-Bm*R1/*Ll-Bm*R1 using a panel of 262 serum samples. *Bm*R1 is the antigen expressed by *Bm17DIII DNA* sequence; while *Ov-Bm*R1/*Ll-Bm*R1 is the antigen expressed by the homolog of *Bm17DIII* DNA in *O. volvulus* and *L.loa.*

Type of serum sample	No	*Bm*R1 (%)	*Ov-Bm*R1/ *Ll-Bm*R1 (%)
O. volvulus, mf positive	70	1 (1.43)	1 (1.43)
L. loa, mf positive	14	6 (42.86)	6 (42.86)
W. bancrofti, mf positive	33	8 (24.24)	8 (24.24)
B. malayi, mf positive	28	28 (100)	28 (100)
Trichuris trichiura	8	0	0
Ascaris lumbricoides	8	0	0
Mixed infection with *T. trichuris*, *A. lumbricoides* and hookworm	8	0	0
Entamoeba histolytica (invasive)	11	0	0
Toxocara	14	0	0
Gnathostoma spinegerum	1	0	0
Strongyloides stercoralis	6	0	0
Endemic normals	29	0	0
Non-endemic normals	32	0	0
TOTAL	**262**		

Reactivities of *Bm*R1 and its *O. volvulus/L. loa* homolog with serum antibodies of the other three IgG subclasses (IgG1, IgG2 and IgG3) using samples from *O. volvulus* and *L. loa* infected individuals showed positive reactions with only IgG1. Most IgG1 positive samples showed OD >1.000. Similarly, the reactivities of anti-filarial IgG1, IgG2 and IgG3 antibody subclasses with *Bm*R1 on serum samples from active and chronic cases *W. bancrofti* and *B. malayi* showed positive reactions only with IgG1. It is also noted that sera from non-endemic normals and soil-transmitted infections also showed similar reactivities i.e. IgG1 positive and IgG2 & IgG3 negative (Table 2).

TABLE 2 Results of ELISAs to detect IgG1, IgG2 and IgG3 anti-filarial antibodies in various kinds of serum samples using *Bm*R1 and *Ov-Bm*R1/*Ll-Bm*R1. Both antigens (tested separately) demonstrated identical results with all serum samples.

Type of serum sample	Number of positive results per number of samples tested		
	IgG1-ELISA	IgG2-ELISA	IgG3-ELISA
O. volvulus mf+	47/47	0/21	0/21
L. loa mf+	14/14	0/14	0/14
W. bancrofti mf+	6/6	0/6	0/6
W. bancrofti chronic	6/6	0/6	0/6
B. malayi mf+	10/10	0/10	0/10
B. malayi chronic	14/14	0/14	0/14
Non-endemic normals	10/10	0/10	0/10
Soil-transmitted helminth infections	10/10	0/10	0/10

Discussion

*Bm*R1, a recombinant *B. malayi* antigen of ~ 30 kDa expressed by *Bm*17DIII DNA coding sequence (cds), has been consistently shown to be a sensitive and specific antigen for immunodiagnosis of brugian filariasis in studies employing either immunochromatographic rapid test (Brugia Rapid) or ELISA (Rahmah *et al.,* 2003a; Rahmah *et al.,* 2003b). Multicenter evaluations performed on Brugia Rapid showed variable reactivity of *Bm*R1 to sera of *W. bancrofti* infected patients. Reactivity to sera from Chennai, India was 54.5% (12/22); from Indonesia was 70% (14/20) and from Cook Island was 90% (9/10) (Rahmah *et al.,* 2003a). The wide variation in the reactivity of the assay in Bancroftian filariasis in the above three geographical areas prompted us to undertake the current investigation. The present study has shown that the homolog in *W. bancrofti* is 100% similar to the cDNA of *Bm*R1, irrespective of the source of the parasites- whether the mf were isolated from the individuals whose sera showed positive or negative reactivity with the *Bm*R1 rapid test. Thus the observed differences in the reactivity of *Bm*R1 antigen with *W. bancrofti* sera collected from different geographical regions does not appear to be due to genotypic variability between different isolates of mf.

PCR experiments were repeatedly performed on the *W. bancrofti* genomic DNA samples to obtain an amplicon with a size greater than 618 bp since an intron is expected to be present in genomic material. However only one prominent band of 618 bp was obtained; and very occasionally a faint band of >1kb was observed which later was shown to be due to unspecific amplification. PCR on *W. bancrofti* genomic DNA to amplify the intron sequence (using primers based on *Bm17DIII* intron) produced a sequence that is ~75% similar to intron of *Bm17DIII.* This is believed to be amplification on another part of *W. bancrofti* genome since PCR using a pair of internal primers that flank the possible intron site produced a PCR product of ~300 bp, a size that is expected if there was no intron. On the other hand amplification of *B. malayi* genomic material produced two kinds of amplicons i.e. 618 bp and 1010 bp. The latter comprised an intron (393bp) and two flanking exons (237bp and 381bp); the sequences were consistent with the *B. malayi* data at TIGR website. Thus at USM, genomic DNA of *Wb17DIII* was found to be intronless; whereas genomic DNA of *Bm*17DIII was shown to have two variants i.e. one with and one without an intron. These results, though seemingly controversial, were a result of exhaustive effort with proper PCR controls. Reports from other laboratories will hopefully confirm these results.

Anti-*Bm*R1 IgG4 was detected in 84.6% (44/52) of *L. loa* sera but generally not detected in *O. volvulus* serum samples (Rahmah *et al.,* 2003b; Fischer *et al.,* in press). The *Bm*R1 homologs of *O. volvulus* and *L. loa* were 100% identical to each other and 99.7% similar to the *B. malayi* and *W. bancrofti* homolog on the nucleotide level (Figure 1). The recombinant *O. volvulus/ L. loa Bm*R1 homolog was found to display a 100% similar reactivity compared to *Bm*R1 when tested with IgG4-ELISA on a panel of serum samples (Tables 1 & 2). Therefore, the difference of one amino acid between the *O. volvulus/ L. loa* and the *B. malayi/ W. bancrofti Bm*R1 homologs did not alter their antigenicity. It is interesting to note that although IgG4 assays have been shown to be elevated in onchocerciasis in assays using other recombinant antigens (Lucius *et al.,* 1992), the IgG4 reactivity to *Bm*R1 or *Ov-Bm*R1 in *O. volvulus* was generally negative. One possible explanation is that the *Ov-Bm*R1 is mostly expressed by adult worms, and the immune response to *O. volvulus* is predominantly due to mf (Kazura *et al.,* 1993), this may explain the very poor IgG4 response to *Bm*R1/ *Ov-Bm*R1. It is possible that the uptake of antigen of lymphatic filariae by antigen presenting cells is significantly different compared to *O. volvulus*, where adult worms and mf reside either in subdermal nodules or in the skin.

The *Bm*R1 antigen and the *O. volvulus/ L. loa* homolog were also used to determine if IgG1, IgG2 or IgG3 antibodies in *O. volvulus, L. loa, B. malayi* and *W. bancrofti* sera samples were reactive with the recombinant proteins. In all cases, only anti-filarial IgG1 was reactive; while anti-filarial IgG2 and IgG3 assays were consistently negative. It is important to note that IgG1 antibodies to *Bm*R1 and homologs are unspecific and without any diagnostic value. The *Bm*R1 antigen contains obviously widespread

epitopes which are recognized by IgG1 antibodies.

Based on the present report and previous studies with IgG4 reactivity, *Bm*R1 and its homologs in *W. bancrofti*, *O. volvulus* and *L. loa* induce IgG antibody responses restricted to IgG1 and IgG4 subclasses only. Unlike the anti-filarial IgG4 response in *B. malayi* infection, the IgG4 response to *Bm*R1 in *W. bancrofti* and *L. loa* was not consistently detected in all infected individuals indicating that this recombinant antigen will not be of much utility for diagnosis of these two filarial infections. Although IgG1 response to *Bm*R1 was observed in all the filarial infections tested, it lacks specificity since it was also positive when tested with serum samples from normal individuals and with those harbouring other parasitic infections.

The study demonstrates the presence of identical/almost identical homologs of the diagnostic *Bm*R1 antigen in other filarial parasites, but they do not seem to induce consistent antibody responses in all infected subjects. Thus the immunogenicity of *Bm*R1 in brugian filariasis appears to be clearly different from that of bancroftian filariasis, onchocerciasis and loiasis.

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