

ORIGINAL ARTICLE

Immunolocalization and serum antibody responses to *Brugia malayi* pepsin inhibitor homolog (Bm-33)

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ABSTRACT

cDNA coding for *Brugia malayi* pepsin inhibitor homolog (Bm-33) from the human filarial parasite was cloned in pRSET for large-scale expression and functional characterization. The pRSET-B cloned gene did not yield recombinant protein expression and the reason was attributed to the presence of an N-terminal signal peptide. The gene was subcloned in pRSET-A without a signal peptide and the 33 kDa histidine-tagged recombinant protein was purified by IMAC. All individuals from an endemic area generated IgG responses against Bm-33 in the order MF>CP>EN. Isotype analysis indicated an elevated IgG4 reactivity in the order MF>EN>CP. Bm-33-specific IgE levels were elevated in MF, CP and EN compared to non-endemic normals with no significant differences among the groups. Paraffin-embedded sections of *Setaria digitata* (cattle filarial parasite) stained with mouse anti-Bm-33 antibodies exhibited the hypodermal nature of Bm-33. These findings suggest that Bm-33 is an immunodominant antigen and contributes to filarial pathogenesis.

Key words antibody, *Brugia malayi*, ELISA, immunolocalization, isotype.

Human lymphatic filariasis is caused by lymph-dwelling, parasitic nematodes such as *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* which are prevalent in tropical and subtropical countries of the world. The initiatives of the Filarial Genome Project to combat this nematode scourge have enabled the identification and characterization of genes with diagnostic and prophylactic capabilities such as WbSXP-1, Bm-ALT etc. (1, 2). Despite these and a few more, there is an urgent need to identify and characterize more antigens involved in parasite-specific immune modulation, a major factor that contributes to filarial pathogenesis.

The cDNA coding for *B. malayi*, pepsin inhibitor homolog (Bm-33), a 33 kDa protein (3, 4), was cloned

and serologically characterized. Earlier, a similar homolog from *Onchocerca volvulus* (Ov33) was cloned in pGEX-2T and the GST tagged-recombinant protein was used for immunodiagnosis based on its elevated IgG4 reactivity in microfilaremic patients (5, 6). Homologs were also identified from related parasites such as *Dirofilaria immitis* and *Acanthocheilonema viteae* and the respective recombinant proteins (Di33 and Av33) were used for the development of immunodiagnostic assays (7, 8). In the case of the Brugian homolog, the 750 bp cDNA coding for Bm-33 was cloned previously in M13mp18. Besides this, preliminary serological analysis was carried out with the λ gt11 expressed, β -galactosidase fusion protein derived from the Bm11-1-6 clone (3) and not a pure protein. In

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List of Abbreviations ALP, alkaline phosphatase; anti-BmA, antibodies against *Brugia malayi* crude protein; BCIP, bromochloroindolyl phosphate; CP, chronic pathology; DAB, diaminobenzidine; EN, endemic normal; HLA, human leukocyte antigen; HRP, horseradish peroxidase; IgG, immunoglobulin G; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl beta thiogalactosidase; MF, microfilaremic; NBT, nitroblue tetrazolium; NEN, non-endemic normal; pNPP, para nitrophenol phosphate.

this regard, 71% and 10–17% reactivities were reported with bancroftian and onchocercian sera respectively, but there were no attempts to assess Bm-33-specific antibody levels across filarial patient groups. In a different study, cloning of Bm-33 in pMAL vector and isotype antibody responses in only EN and MF patients of Indonesian origin was demonstrated (4). Thus, to date, no information is available, pertaining to complete serological characterization of recombinant pure Bm-33, particularly with filarial patients of Indian origin. The present paper describes the production of pure recombinant Bm-33, its reactivity with filarial patient sera, by examining the IgG isotypes and IgE distribution, along with epitope analysis and immunolocalization in *Setaria digitata*. The cattle filarial parasite deems usage as a model for human filarial parasite in the current study, as it is a rich source of parasite material and exhibits cross-reactivity with bancroftian sera (9).

MATERIALS AND METHODS

Cloning of the gene coding for Bm-33

The cDNA (702 bp) coding for Bm-33 was cloned from *B. malayi* L-3 cDNA library in TOPO plasmid (Invitrogen, San Diego, CA, USA). This was cloned into pRSET-B between BamHI and EcoRI restriction sites according to standard protocols (10) which did not yield recombinant protein expression. Hence, the cDNA region, away from the signal peptide coding region was PCR amplified and the 645 bp insert was cloned into pRSET-A.

Nucleotide sequence analysis of Bm-33

pRSET-B-Bm-33 plasmid was sequenced at Microsynth, Balgach, Switzerland and the nucleotide sequence was analyzed by BLAST at www.nematodes.org.

Multiple sequence alignment of the obtained Bm-33 nucleotide sequence, along with sequences, [L11001](#) (*B. malayi* pepsin inhibitor homolog), [AI066036](#) and [AI723728](#) from *B. malayi* microfilaria and L-3 stages respectively, was carried out by Clustal W using the DNA STAR, MEGA ALIGN program (Lasergene).

Recombinant Bm-33 protein expression

Transformed BL21 (DE3) colonies were screened for recombinant protein expression by inducing the *E. coli* cultures with 0.1 mM IPTG (Calbiochem, San Diego, CA, USA) at 37°C for 3 hr. The protein profiles of uninduced and induced cultures along with the host (BL21 [DE3]) and vector control (BL21DE3+pRSET-A without insert) were analyzed on 12% SDS-PAGE (11).

Western blot analysis

SDS-PAGE-resolved protein was transferred onto a nitrocellulose membrane (Hybond; Amersham Pharmacia, Sunnyvale, CA, USA) using a semi-dry blotting apparatus (Amersham Pharmacia) at a constant voltage of 20 volts and 150 milliamps for 1.5 hr. The membrane was blocked for 2 hr at 37 °C using 5% skimmed milk powder in 1× PBS and washed thrice in 0.05% Tween-20 in PBS/PBS for 10 min. The membrane was subsequently incubated with mouse anti-histidine monoclonal antibody (1:20 000) (Amersham Pharmacia) overnight at 4 °C. After washing the membrane as mentioned above, it was incubated with goat antimouse IgG-ALP (1:20 000; alkaline phosphatase; Sigma-Aldrich, St Louis, MO, USA) for 1.5 hr. Finally, the blot was developed with 33 µl BCIP and 66 µl NBT (USB, Cleveland, Ohio, USA).

Large-scale expression and purification of recombinant Bm-33 by IMAC

Large-scale expression of recombinant protein was carried out by a shake flask method of IPTG-induced cultures. The harvested pellet was dissolved in binding buffer (200 mM NaCl, 100 mM Tris, 100 mM NaH₂PO₄ at pH 8.0) and sonicated at 50 Hz for 12 cycles. The cell lysate was solubilized further by the addition of 8 M urea (Merck Scientific, Darmstadt, Germany) and 1 mg/ml lysozyme (SRL, Mumbai, India). Subsequently, 2–2.5 ml of soluble fraction from the sonicated cell lysate was loaded onto the column of chelating sepharose (Amersham Pharmacia, Sunnyvale, CA, USA) charged with 6% nickel. Protein was eluted using an imidazole gradient (100–200 mM) and the fractions were analyzed on 12% SDS-PAGE. The imidazole fractions were dialyzed overnight against 0.1× PBS at 4 °C and the protein concentration was estimated by the Bradford assay. The aliquots were finally stored at –20 °C.

Study population

Serum samples were collected from individuals residing in and around Chennai, an area endemic to bancroftian filariasis. Patients were recruited through the Filariasis Control Unit under the Directorate of Public Health (Chennai, India) after obtaining informed consent with protocols approved by the Institutional Review Board of Anna University (Chennai). Twenty-five patients from each group: endemic normals (clinically asymptomatic and amicrofilaremic, EN), microfilaremic (clinically asymptomatic, microfilaremic, MF) and chronic pathology (clinically symptomatic and amicrofilaremic, CP) having chronic irreversible lymphedema of the extremities were included in the study (Table 1). Patients were classified

Table 1. Demographic details of patients used in the present study

Group	No. of		Median age (years)	Clinical manifestation	Treatment	Parasite load (AU/ml)	CFA (Og4C3)
	Males	Females					
EN (25)†	12	13	24–35	None	None	None	None
MF (25)	13	12	18–35	None	None	500–1000	(7000–16 000 U)
CP (25)	13	12	20–45	Grade II–IV lymphedema	DEC, heat therapy	None	None
NEN (25)	11	14	20–40	None	None	None	None

†Numbers in parentheses are total numbers of patients.

AU, antigenic unit; DEC, diethylcarbamazine; GM, geometric mean; Og4C3, *Onchocerca gibsoni* antigen.

according to the microfilarial load and assessment of circulating filarial antigen levels (CFA) by ELISA (Og4C3), a marker of *W. bancrofti* infection and adult worm burden (12), (TropBio, Townsville, QLD, Australia). The NEN serum samples that were used in the study were a kind gift from Dr Thomas B. Nutman (NIH, Bethesda, MD, USA).

Assessment of Bm-33-specific antibodies by Western blot with filarial patient sera

The protein (25 µg) was resolved on 12% SDS-PAGE and transferred onto a nitrocellulose membrane as mentioned earlier. The membrane was cut into strips and probed with NEN, EN, MF and CP sera (1:100). A strip probed with mouse anti-histidine monoclonal antibody was used as a positive control and another strip was probed with rabbit anti-BmA (1:200) (rabbit antiserum against *B. malayi* crude antigen preparation). Subsequently, the strips probed with patient serum, anti-BmA and anti-histidine were incubated with ALP conjugates (1:20 000) of goat antihuman IgG, goat antirabbit IgG and goat anti-mouse IgG (Sigma-Aldrich), respectively, and were finally developed with NBT-BCIP.

Bm-33 antigenicity and epitope analysis

Proppred analysis was carried out at the default setting (threshold value of 3.0) using the server (<http://www.imtech.res.in/raghava/propred/>) to identify the most promiscuous HLA class-2 binding epitopes of Bm-33.

IgG isotype and IgE levels by ELISA

Protein (5 µg/ml) in coating buffer (0.045 M NaHCO₃ and 0.02 M Na₂CO₃, [pH 9.6]) was coated onto immulon-4 micro titer ELISA plates, based on the checker board titration method and incubated for 3 hr at 37 °C. The plates were washed thrice with 0.05% Tween-20 in 1× PBS in an automated ELISA washer and were blocked with 5% bovine serum albumin (Amersham Pharmacia) for 2 hr at 37 °C. Serum (100 µl) from NEN, EN, microfilaremic

and CP patients (1:100 in 1× PBS) was added to the plate and incubated overnight at 4 °C. The plates were washed in PBS and incubated with a 1:20 000 dilution of goat antihuman IgG-ALP conjugate for 2 hr. The plates were finally developed with 1 mg/ml pNPP in sodium carbonate buffer and the absorbance was read in an ELISA reader (Multiscan; Thermo Fischer Scientific, Vataa, Finland) at 405 nm. The absorbance of the wells coated with PBS alone was subtracted from all values. Control wells containing all reagents except serum were also used in each plate.

Bm-33-specific, serum isotype and IgE reactivity among the groups was carried out with the same samples. After serum incubation and wash, plates were incubated with isotype-specific monoclonal antibodies (anti-IgG1 clone HP-6069, anti-IgG2 clone HP-6002, anti-IgG3 clone HP-6047 and anti-IgG4 clone HP-6023, monoclonal antibodies to human IgE) at a dilution of 1:1000, except anti-IgG2, which was added at a dilution of 1:3000. The plates were subsequently incubated for 2 hr at 37 °C with ALP-conjugated goat antimouse IgG (Fc specific) (Jackson ImmunoResearch, Baltimore, MD, USA). Finally, the plates were developed with pNPP (Thermo Fischer Scientific, Rockford, IL, USA) as mentioned earlier and absorbance was determined at 405 nm using an ELISA reader.

Immunolocalization

Setaria digitata male and female worms were obtained from a local abattoir in Chennai from the peritoneal cavity of cattle. The worms were washed gently by agitation in 1× PBS for 30 min, by changing the PBS at 10-min intervals. The slides containing paraffinized sections were de-paraffinized by immersing them in three changes of xylene followed by 100% ethanol for 3 min each. The sections were rehydrated by placing the slides in 95%, 80% and 70% ethanol for 2–3 min followed by 1× PBS for approximately 5 min. Finally, the slides were incubated in 300 ml of 3% H₂O₂ diluted from a stock solution of 30% with 1× PBS for 10–15 min to remove the endogenous peroxidases in the sections that could give a false-positive signal.

The sections were dried and blocked in 5% BSA for 1 hr and washed thrice with 1× PBS for 5 min each. Subsequently, they were incubated in mouse anti-Bm-33 and normal mouse serum (1:1500) for 2 hr at room temperature followed by three washes with 1× PBS for 5 min each. The dried slides were incubated in goat antimouse IgG horseradish peroxidase conjugate (1:20 000) (Sigma-Aldrich) for 1 hr at room temperature. The slides were finally washed thrice with 1× PBS for 5 min and developed with 6 mg DAB (Sigma-Aldrich) in 12 ml of 0.1 M Tris (pH 7.2) and 6 μ l H₂O₂. Following incubation for 5–10 min the slides were washed with PBS to stop the

reaction. The dried slides were mounted with DPX mountant (Merck) and visualized under 400× by a compound microscope (Olympus, New York, NY, USA).

Statistical analysis

All statistical analyses pertaining to the absorbance values in ELISA were carried out using Graph Pad Prism software. The comparison between groups were done using the Kruskal–Wallis test followed by Dunn's post-hoc multiple comparisons. A *P* value of *P* < 0.05 was considered significant.

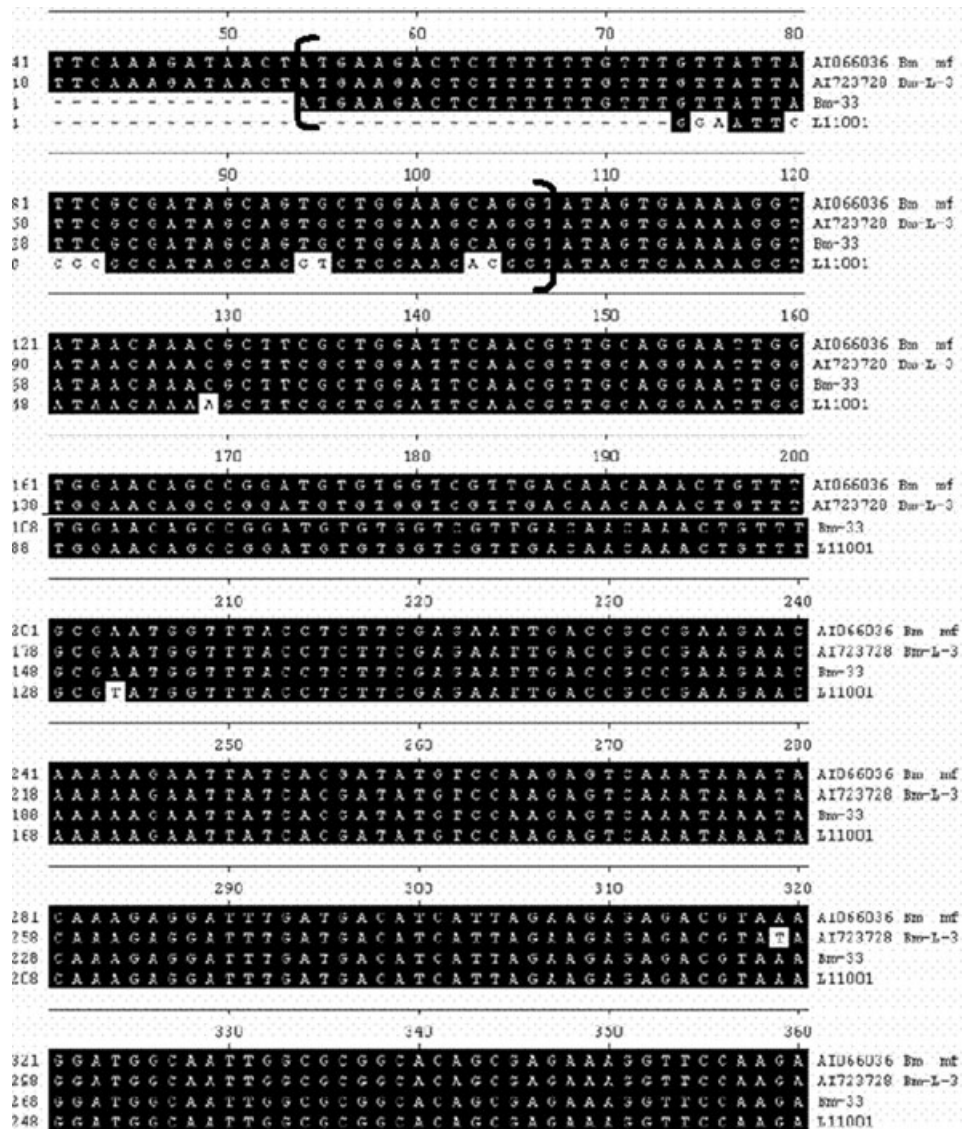


Fig. 1. Multiple sequence alignment of Bm-33 with identical sequences in a database from *B. malayi* mf (AI066036), L-3 (AI723728) and adult male (L11001) stages. Region coding for signal peptide is enclosed in brackets.

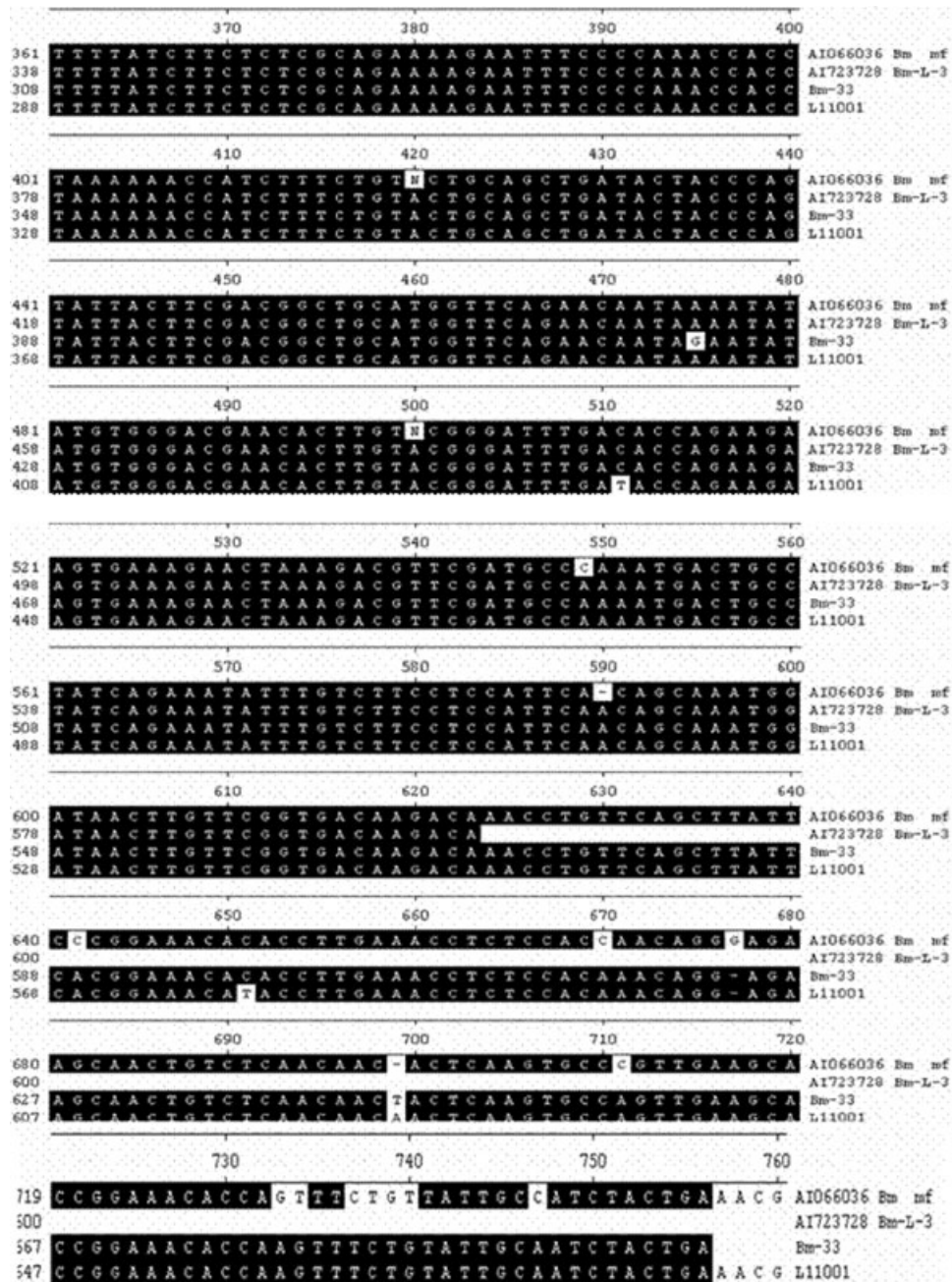


Fig. 1. Continued.

RESULTS

Expression of recombinant Bm-33

Multiple sequence alignment of Bm-33 indicated a >98% similarity at the nucleotide level with sequences in the database from *B. malayi* adult male, (L11001),

microfilaria (AI066036) and L-3 stage (AI723728) (Fig. 1).

pRSET-A-cloned Bm-33 resulted in the expression of a 33 kDa protein in the uninduced and induced cultures of BL21 (DE3) on 12% SDS-PAGE in comparison to controls (Fig. 2a). Western blotting with monoclonal antibody to histidine showed similar observations (Fig. 2b).

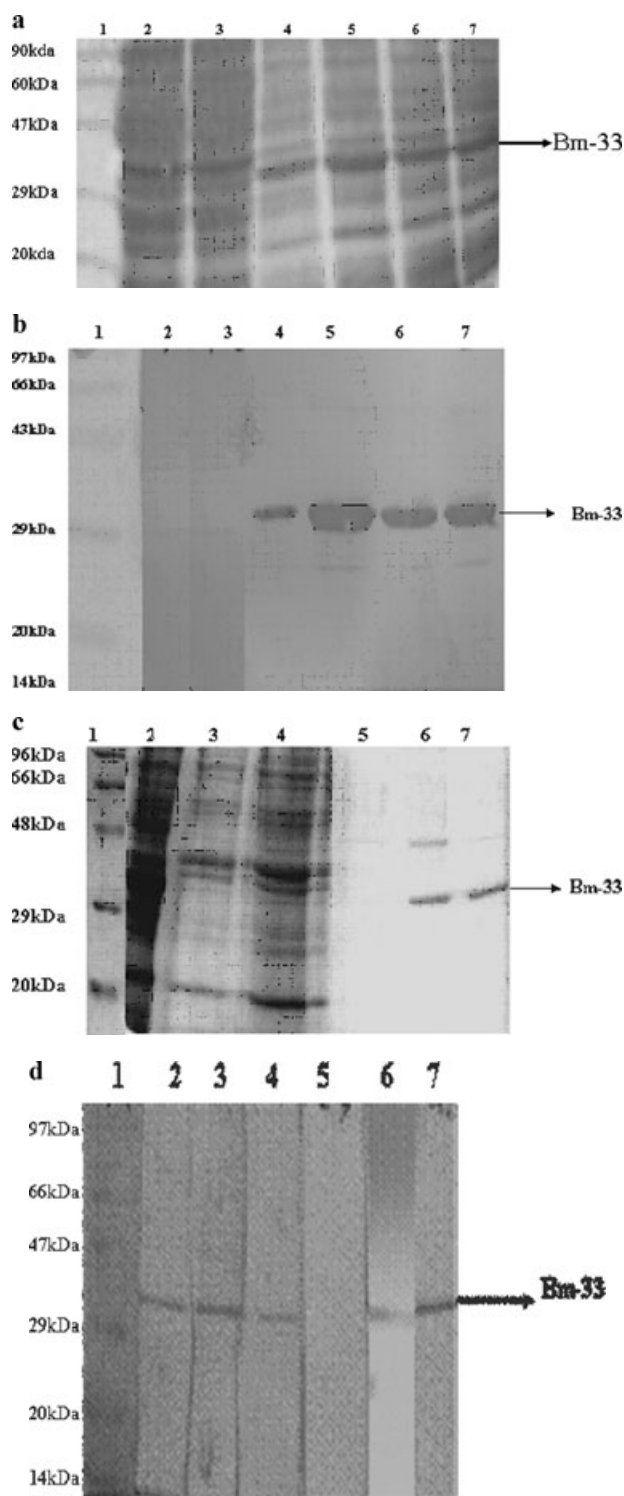


Fig. 2. (a) Twelve percent SDS-PAGE showing the IPTG-induced expression profile of recombinant Bm-33. Lane 1, protein molecular weight marker; Lane 2, BL21(DE3) induced with 0.1 mM IPTG; Lane 3, BL21(DE3)-pRSET-A induced with 0.1 mM IPTG; Lane 4, uninduced; Lanes 5, 6 and 7, colonies 1, 2 and 3 induced with 0.1 mM IPTG.

Purification and characterization of recombinant Bm-33

Twelve percent SDS-PAGE analysis of IMAC fractions showed Bm-33 as a single band in the protein fractions eluted with 200 mM imidazole (Fig. 2c). Western blot of IMAC-purified Bm-33 exhibited a similar reactivity with the patients' sera including EN, except from those of non-endemic normals (Fig. 2d). The reactivity with MF and CP were more prominent compared to EN.

Bm-33-specific IgG, isotype and IgE reactivity with patients' sera by ELISA

Bm-33-specific IgG levels were significantly high in MF compared with other groups ($P < 0.05$) and in the order MF > CP > EN > NEN (Fig. 3a).

Recombinant Bm-33-specific IgG4

Recombinant Bm-33-specific IgG4 levels (Fig. 3b) were significantly higher in MF compared with CP and EN in the order MF > EN > CP > NEN ($P < 0.05$). However, there was no significant difference observed with respect to IgG4 levels in EN and CP ($P > 0.05$). Similarly, elevated levels of Bm-33-specific IgG1 were observed in MF compared with CP and EN, in the order MF > CP > EN > NEN. Nevertheless, IgG4 exhibited a higher reactivity in MF compared with IgG1 ($P < 0.05$). In contrast, recombinant Bm-33-specific IgG2 and G3 levels were elevated in CP and EN compared to MF in the order CP and EN > MF > NEN ($P < 0.05$).

Recombinant Bm 33-specific IgE

Bm-33-specific IgE levels were elevated in EN, MF and CP compared to the non-endemic normals ($P < 0.05$). There

(b) Western blot with monoclonal antibody to histidine confirming the expression of pRSET-A-Bm-33. Lane 1, molecular weight marker; Lane 2, IPTG-induced BL21(DE3) host; Lane 3, IPTG-induced BL21(DE3)+ pRSET-A-Bm-33; Lane 4, uninduced BL21(DE3)+ pRSET-A-Bm-33; Lanes 5, 6 and 7, induced BL21(DE3)+pRSET-A-Bm-33.

(c) Twelve percent SDS-PAGE showing recombinant Bm-33 purification by IMAC. Lane 1, protein molecular weight marker; Lane 2, induced Bm-33 cell lysate; Lane 3, flow through; Lane 4, wash; Lane 5, 100 mM imidazole eluted fraction; Lane 6, 150 mM imidazole eluted fraction; Lane 7, 200 mM imidazole eluted fraction. (d) Western blot showing the reactivity of recombinant pure Bm-33 with patient sera. Lane 1, molecular weight marker. Lanes 2–5, show differential reactivity with sera (1:100) dilution from different filarial clinical groups (Lane 2, CP; Lane 3, MF; Lane 4, EN; Lane 5, NEN); Lane 6, rabbit anti-BmA (1:200); Lane 7, monoclonal mouse anti-histidine.

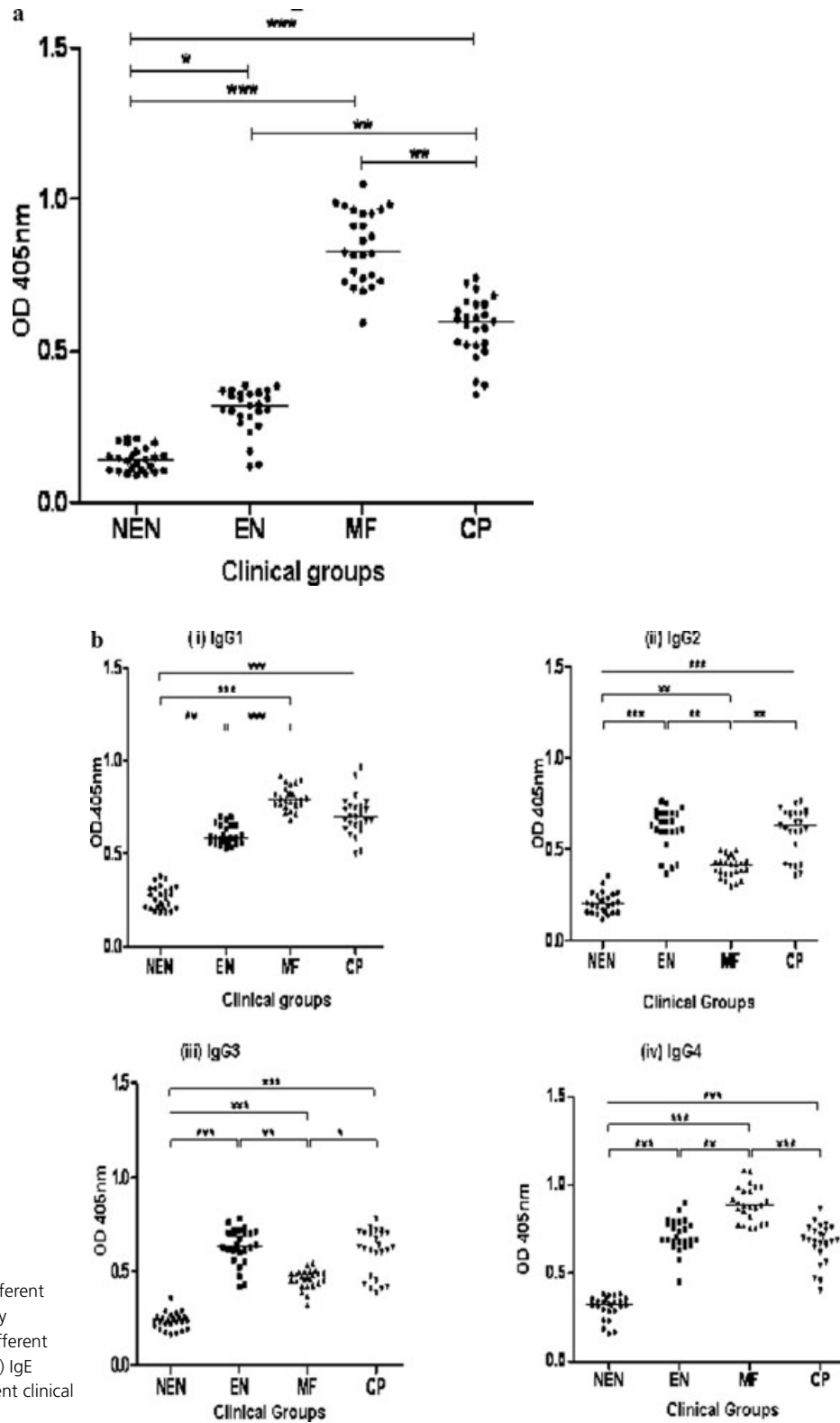


Fig. 3. (a) Anti-Bm-33 IgG levels in different clinical groups. (b) IgG isotype antibody responses to recombinant Bm-33 in different clinical groups determined by ELISA. (c) IgE antibody responses to Bm-33 in different clinical groups determined by ELISA.

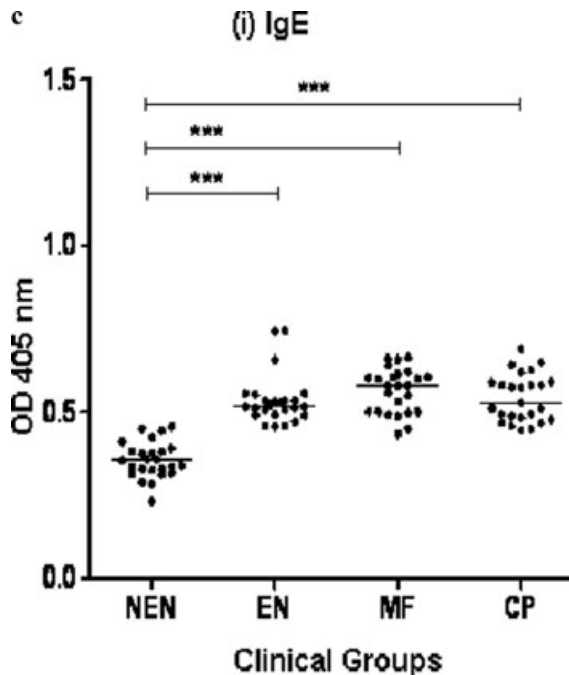


Fig. 3. Continued.

was no significant difference in Bm-33-specific IgE levels between the patient's sera and endemic normals (Fig. 3c). However, Bm-33-specific IgG4 was high compared with Bm 33-specific IgE in the order MF>EN>CP ($P < 0.05$).

Bm-33 epitope and antigenicity analysis

Propred analysis revealed the presence of nanomers binding to HLA DR alleles (32 out of 51) with a binding score >50% (Table 2). In this regard, although many peptides from Bm-33 that bound to other HLA class-II alleles were observed, only those peptides that had a binding score of more than 50% were reported.

Immunolocalization

Structural organization of the deparaffinized sections of both male and female *S. digitata* worms, stained with hematoxylin and eosin, showed the general body wall organization of nematodes with respect to the outer cuticle, hypodermis, lateral chords, musculature and viscera. Localization studies with mouse anti-Bm-33 demonstrated reactivity on the surface as well as on the inner regions corresponding to the musculature and viscera in both male and female worms (Fig. 4b and d), but the same reactivity was absent in similar sections probed with normal mouse serum under the same conditions (Fig. 4a and c).

Table 2. Propred analysis showing HLA class-II binding epitopes of Bm-33

Serial no.	Sequence	Position	No. HLA alleles
1	VKRYNKRFA	1	9
2	VVVDNKLFA	23	8
3	YVQESNKYK	50	2
4	YYFDGCMVQ	111	4
5	YVGRTLVRD	124	2
6	VGRTLVRDL	125	2
7	LVRDLTPPE	129	2
8	YQKYLSSS	151	1
9	LFGDKTNLF	166	2

Predicted T-cell epitopes of Bm-33 which bind to HLA class-II alleles with maximum probability.

DISCUSSION

Cloning of Bm-33 (645 bp) in pRSET-A without an N-terminal signal peptide (57 bases) coding region was an alternative strategy for recombinant protein expression, as there was no protein expression from pRSET-B clone. This was obvious as the pRSET-A clone resulted in a 33 kDa protein expression upon IPTG induction, unlike the pRSET-B clone that showed no expression, even with alternative *E. coli* hosts such as GJ1158 (13) and BL21(DE3) pLysS (14) (data not shown). Western blotting results support this view. The >98% similarity between Bm-33 nucleotide sequences from different developmental stages of the parasite suggests that Bm-33 might not be a stage-specific antigen. This is further evident from the fact that previous studies demonstrated the identification of Bm-33 cDNA from adult male stages of the parasite (3, 4) and the current work from the L-3 stage of the parasite.

An interesting observation was the expression of the 33 kDa protein by an insert cDNA of 645 bp. However, previous reports mention the use of an identical recombinant protein from *Brugia* as well as from other homologs such as *Dirofilaria* and *Onchocerca* (Di-33, Ov-33). These observations (15–17) exemplify the fact that the N-terminal signal peptide presented problems in recombinant protein expression and the mature protein following signal peptide cleavage exhibited a retarded mobility.

The preliminary serological characterization of IMAC-purified Bm-33 by western blotting with patient serum and anti-BmA antibodies confirms protein origin and demonstrates that signal peptide removal does not affect protein reactivity. The observations pertaining to differential serum reactivity of Bm-33 by western blotting support the findings of ELISA at the IgG level. The absence of reactivity with NEN in both cases suggests that NEN may not have antibodies against Bm-33. Similar observations

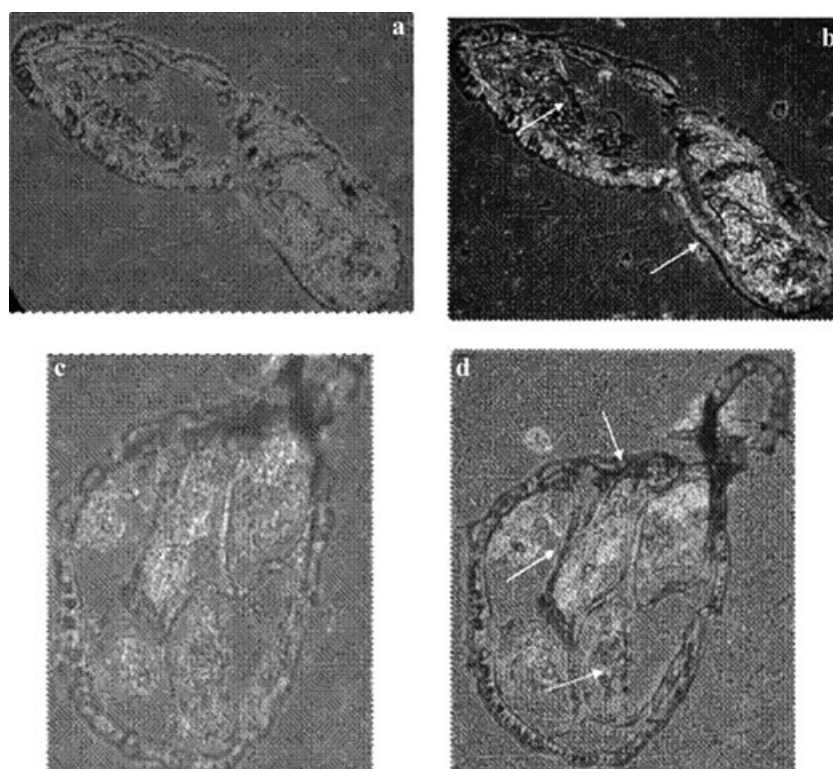


Fig. 4. Immunolocalization of Bm-33 on paraffin-embedded sections of *Setaria digitata*. Microscopic images ($\times 400$) of male (a,b) and female (c,d) *Setaria digitata* paraffin-embedded sections probed with mouse anti-Bm-33 (b,d) and normal mouse serum (a,c), followed by goat anti-mouse IgG-HRP. The sections were developed with DAB and visualized. Localization was observed in the outer hypodermis and internal viscera comprised of muscle fibers along with ovarian tissue and the female reproductive tract (arrows). The sections probed with normal mouse serum do not exhibit similar reactivity.

pertaining to MF and CP were recorded with other homologs, such as Di-33 and Ov-33, where the protein was used for immunodiagnosis based on its elevated reactivity in prepatent and occult cats and dogs (15). Animal studies with Ov-33 suggested that detectable antibodies were observed during the course of parasite maturation and production of microfilariae (5) which imply the MF stage of patients.

Bm-33 epitope analysis corroborates the findings pertaining to serum antibody reactivity. The presence of HLA-2-binding peptides in Bm-33 implies a T-cell stimulating ability of the protein, which is important for the induction of the antibody and isotype responses. Similar assessment of HLA-2 binding peptides identification was carried out in a different study by Propred analysis (18).

Bm-33 exhibits an elevated reactivity at the IgG4 and IgG3 isotypes, respectively, as they are the most predominant isotypes in MF and CP (19). Elevated Bm-33-specific IgG3 in CP may contribute to pathology by tissue damage. The role of IgG3 in type III immunogenic reactions leading to tissue damage has been reported previously in CP patients (20). Thus, elevated reactivity in these groups at the respective isotype levels implicates the role of Bm-33 in immunopathogenesis associated with these groups. Elevated IgG4 and IgG1 reactivity in MF and EN was, however, demonstrated as part of a different study (4) on

patients from Indonesia, where the reactivity was not assessed in the CP group. Elevated Bm-33-specific IgG2 in CP and EN compared to MF may imply a protective role of the antigen, as IgG2 antibodies are involved in anti-microfilarial and anti-larval immunity against carbohydrate determinants on the sheath (21–23). In this regard, it would be interesting to identify the post-translational modifications in Bm-33, as IgG2 responses are primarily directed against carbohydrate antigens and proteins with repetitive structures (24, 25). Further, an assessment of cytokine and costimulatory molecule expression in response to Bm-33 across various clinical groups will enable a better understanding of the protective nature of recombinant protein that is currently in progress. Besides the current study, similar observations pertaining to parasite recombinant protein-specific diminished IgG2 in MF compared to CP and EN were reported previously (26). An elevated Bm-33-specific IgG4 in the EN is interesting, as this is a marker for active filarial infection (27). This may be due to the fact that the classification of EN is controversial in filariasis as many, if not all, may harbor cryptic infections (28). Further, elevated filarial-specific IgG4 in EN was demonstrated as part of a different study (29). An elevated Bm-33-specific IgG4 reactivity in MF, CP and EN corroborate with the elevated IgE reactivity, suggesting that IgG4 and IgE may share a common antigenic epitope.

Similar findings pertaining to IgG4 and IgE production from peripheral blood mononuclear cell supernatants was reported in a different study with OvD5B, an analog of Ov-33 (30). Besides this, diminished Bm-33-specific IgE/IgG4 in MF indicates the probable role of this parasite antigen in generating blocking antibodies in this group (31). A similar observation in CP and EN is interesting and needs to be assessed by further experimentation.

Immunolocalization studies augment the immunodominant nature of Bm-33 evidenced by elevated serum antibody levels across all the groups. The visceral reactivity in female and male sections of *S. digitata* demonstrates the presence of Bm-33 in muscles and the reproductive system. Besides this, reactivity on section margins imply a hypodermal nature of the antigen which makes Bm-33 a potent candidate for stimulating host immune elements. Similar observations pertaining to localization were observed with related homologs such as Ov-33 (5). The importance of immunolocalization studies towards the assessment of an antigen's ability to stimulate host immune elements can be gauged by our findings on WbSXP-1. In this regard, immunolocalization studies on *S. digitata* with anti-WbSXP-1 demonstrated the hypodermal nature of SXP-1 (32), which was further substantiated as an immunodominant antigen by monitoring its effect on T-cell and accessory cell functions (33).

Thus, the current study suggests Bm-33 to be an immunodominant and hypodermal antigen that predominantly induces an IgG4 response as observed by immunolocalization and ELISA. However, it can be envisaged from the current work that serum antibody responses to the parasite antigen depends on modulating factors such as the presence of active or cryptic infections which may, at times, throw up indecisive or overlapping findings that do not define its exact role. However, these findings can provide interesting leads to filarial pathogenesis when corroborated with the Bm-33-specific cytokine and costimulatory molecule expression across filarial patients. Such studies are being pursued in our laboratory.

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