Association between Mannose-Binding Lectin Polymorphisms and *Wuchereria bancrofti* Infection in Two Communities in North-Eastern Tanzania

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Abstract. The association between selected mannose-binding lectin (MBL) genotype polymorphisms and *Wuchereria bancrofti* infection status was assessed among individuals whose infection status had been monitored for three decades. Blood samples were collected in 2006 and examined for polymorphisms in the *mbi-2* gene and for *W. bancrofti*-specific circulating filarial antigen (CFA) status. Logistic regression analysis showed a significant association between MBL genotype and CFA status, with low-expression MBL genotype individuals being almost three times more likely to be CFA positive than high-expression MBL genotype individuals (odds ratio [OR] = 2.90). When individuals' filarial infection (microfilaria) status in 1975 was included in the analyses, the gain of new infections between the two examination points was almost 10 times higher among individuals with low than among those with high MBL expression genotype (OR = 9.51). The susceptibility to *W. bancrofti* infection thus appears to be significantly affected by the MBL expression genotype of the host.

INTRODUCTION

Lymphatic filariasis resulting from infection with the mosquito-borne filarial nematode *Wuchereria bancrofti* affects millions of people in tropical developing countries. It is a cause of severe disability and has important socio-economic consequences.¹ The adult filarial worms reside in the lymphatic system and induce debilitating acute and chronic manifestations such as filarial fever, lymphangitis, limb elephantiasis, and hydrocele. The larvae (microfilariae) circulate in the blood. Mosquitoes pick up the larvae during a blood meal, and after a period of development within the vector, the larvae can infect new human hosts.

Household and familial clustering of *W. bancrofti* infections are commonly observed in epidemiological surveys,^{2–5} and individuals apparently vary in their likelihood to acquire infection. This notion is supported by the very static infection patterns observed in long-term follow-up studies.^{6–9} Clustering of infection could be an effect of individual differences in exposure to infective vectors, which has been shown to vary considerably within the endemic community,¹⁰ or in adaptive immune responsiveness, which e.g. may be affected by the infection status of the mother during pregnancy.¹¹ It is also possible that innate characteristics of the individuals such as genetic factors could play a role, and various attempts have been made to analyze the human genetic composition in relation to both infection susceptibility and development of clinical manifestations.^{2,12–16}

A study in India suggested that polymorphisms in the *mbi-2* gene could affect susceptibility to *W. bancrofti* infection.¹⁷ This gene is encoding for production of mannose-binding lectin (MBL), a collagen-like serum protein, which binds to a variety of sugars on the surface of pathogens and thereby facilitate

innate host defense to invading pathogens.¹⁸ Individual differences in MBL serum concentrations caused by polymorphisms in the *mbi-2* gene, result in allele variants which, according to Garred and others,¹⁹ can be grouped into high- and low-MBL expression genotypes.

During the last three decades, long-term studies on the epidemiology and control of *W. bancrofti* infection have been carried out in a number of selected rural communities of Tanga Region, Tanzania.^{7,20,21} In light of the findings suggesting a link between *mbi-2* gene polymorphisms and susceptibility to filarial infection,¹⁷ we decided to assess the *mbi-2* gene polymorphisms and the corresponding MBL expression genotypes in the remaining individuals from this Tanzanian cohort and to relate these findings to the present and past *W. bancrofti* infection status of the individuals.

MATERIALS AND METHODS

Study communities. The study was carried out in Kwale and Tawalani, two coastal villages located 27 and 38 km north of Tanga town, respectively, in Tanga Region, Tanzania. The villages, which are highly endemic for *W. bancrofti* infection, have previously been described in detail.²¹ Individuals from the two villages who had been examined for microfilariae (mf) in 1975, and who were re-identified in 2006 and gave oral informed consent to participate, were included in this study. Research and ethical clearance was granted by the Medical Research Coordinating Committee of the National Institute of Medical Research (NIMR) in Tanzania, and the study proposal was reviewed by the Danish National Committee for Biomedical Research Ethics.

Previous filariasis activities in the study communities. Cross-sectional surveys for bancroftian filariasis were first carried out in Tawalani and Kwale in 1975,²⁰ during which individuals were examined for mf at daytime following administration of a low dose of diethylcarbamazine (DEC), the so-called DEC provocative day test. This was followed by application of vector control in Kwale and DEC mass treatment in Tawalani,

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and several follow-up surveys were carried out during the next couple of years to evaluate the effect of these measures.^{22,23} Cross-sectional surveys for *W. bancrofti* mf were again carried out in 1991,²¹ this time by examination of night blood specimens, and were followed by further DEC mass treatment in 1992 and a number of follow-up surveys between 1992 and 2001 to evaluate the effect of the intervention (summarized by Meyrowitsch and others⁷). Major efforts were made during the 1992 and 2001 surveys to re-identify the villagers who had participated in earlier surveys (for detailed description of the re-identification procedure, see Ref. 6). More recently, Tawalani and Kwale were included in the Tanzanian National Program for Elimination of Lymphatic Filariasis (NPELF), which offered the inhabitants mass treatment with ivermectin and albendazole in November 2004 and February 2005.

Field sampling in the present study. In July 2006, all individuals examined in 1975 who were still present in the communities were re-identified by using the procedure previously described.⁶ The mass-treatment applied by the NPELF had reduced microfilaraemias, thus making diagnosis of lymphatic filariasis by detection of mf unreliable. Instead, all re-identified individuals were examined for *W. bancrofti*-specific circulating filarial antigens (CFA), which primarily originate from the adult worms and are comparatively less affected by the microfilaricides used in the mass-treatment campaigns. Finger-prick blood samples of approximately 0.5 mL were collected in 1.5 mL EDTA-coated Eppendorf tubes (Eppendorf Nordic, Denmark). The samples were brought back to the laboratory, and after 10 minutes of centrifugation at 2000 rpm the plasma fraction was transferred to another 1.5 mL Eppendorf tube. The two sets of Eppendorf tubes (plasma and blood cell fraction) were frozen at -20° and brought to Denmark for examination for CFA and for analysis of the *mbi-2* gene, respectively.

Examination for circulating filarial antigen. Plasma specimens were examined for CFA by using the TropBio enzyme-linked immunosorbent assay (ELISA) kit (TropBio Ltd. Pty., Townsville, Australia) according to manufacturer's procedures. Specimens with an optical density value greater or equal to standard 2 (32 antigen units) enclosed with the kit were considered positive for CFA. Individuals with a positive or negative test for CFA were defined as filarial positive and negative in 2006, respectively.

Examination for single nucleotide polymorphisms in the *mbi-2* gene. The DNA in the blood cell samples was extracted using the NucleoSpin Blood Quick Pure kit (Macherey-Nagel, Germany) according to manufacturer's instructions. Primer sets M&M1fw + M&M4rv (see Table 1 for sequences) spanning the target region of the *mbi-2* gene containing the polymorphisms at -550, -221, c4, c52, c54, and c57 were selected using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and the *mbi-2* sequence obtained through the NCBI website, product size: 865 bp. The M&M4rv was biotinylated by the manufacturer at the 5'-end (<http://www.MWG-biotech.com>). The cycle parameters were as follows: 95°C, 15 min, 35 cycles of 95°C for 45 sec, 61°C for 45 sec, and 70°C for 90 sec and final extension, 70°C for 10 min. The polymerase chain reaction (PCR) master mixed contained Qiagen HotStarTaq Master Mix Kit (1:1, according to manufacturer's instructions, Qiagen, Denmark), 0.5 µM of each primer and 1 µL of extracted DNA in a final volume of 40 µL.

TABLE 1
Mbi-2 primers and probes for *mbi-2* PCR and SSOP-ELISA*

	Sequence (5' → 3')
Primer name:	
M&M1fw	CCAGGGCCAACGTAGTAAGA
M&M4rv-biotin	TACCTGGTTCCTCCCTTTTCT
Probe target SNP/haplotype:	
MBL2 (-550)-H12	AAG CCT GTG TAA AAC ACC AA
MBL2 (-550)-L12	AAG CCT GTC TAA AAC ACC AA
MBL2 (-220)-X2	ACT GCC ACC GAA AGC ATG
MBL2 (-220)-Y3	TGC CAC GGA AAG CAT GTT
MBL2 (+4)-P	GCA TGC TCG GTA AAT ATG
MBL2 (+4)-Q	GCA TGC TTG GTA AAT ATG
MBL2 (c52 + c54)-AA	TGG GCG TGA TGG CAC CAA
MBL2 (c52 + c54)-DA	TGG GTG TGA TGG CAC CAA
MBL2 (c52 + c54)-AB	TGG GCG TGA TGA CAC CAA
MBL2 (c52 + c54)-DB	TGG GTG TGA TGA CAC CAA
MBL2 (c57)-A	ACC AAG GGA GAA AAG GGG
MBL2 (c57)-C	ACC AAG GAA GAA AAG GGG

*PCR = polymerase chain reaction; SSOP-ELISA = sequence-specific oligonucleotide probe/enzyme-linked immunosorbent assay.

An ELISA method based on the procedure for detection of G6PD-deficiency and sickle cell trait²⁴ was developed to detect the single nucleotide polymorphisms (SNPs) in the *mbi-2* gene. In brief, ELISA plates pre-coated with streptavidin by the supplier (Thermo Fisher Scientific Inc., Denmark) were washed three times in washing buffer (1× PBS with 0.05% Tween-20). The PCR products were denatured at 95°C for 5 min and immediately thereafter cooled to 4°C until used. The 100 µL of cold washing buffer (1× PBS with 0.05% Tween-20) and 2 µL of water-diluted PCR products (1:4) were then added to each well of the ELISA plate. Replicate ELISA plates were made enabling simultaneous probing with sequence-specific oligonucleotide probes (SSOPs) targeting the full panel of *mbi-2* SNPs. The plates were incubated at room temperature for 1 hour and washed three times in washing buffer. The SSOPs were digoxigenin conjugated at the 3-end by the suppliers (<http://www.MWG-Biotech.com>) (for specific sequences, see Table 1). The probes were diluted to 2 nM in tetramethyl-ammonium chloride (TMAC)-solution (3M TMAC [Sigma Aldrich, Germany], 50 mM Tris (pH: 8.0), 0.1% SDS, 2 mM EDTA [pH:8.0]), and added to each plate, 100 µL to each well. The plates were incubated in a thermo shaker oven (PST-60HL-4, BioSan, Riga, Latvia) at 53°C for 1 hour and washed three times in washing buffer. Hereafter followed two rounds of washing and incubation in TMAC solution for 14 min. at 58°C in the oven. To remove TMAC, the plates were washed three times in washing buffer. Peroxidase conjugated anti-digoxigenin antibody in dilution buffer (1:1000) (Roche Diagnostics, Germany) was added to each well and incubated at room temperature for 1 hour. The plates were washed three times in washing buffer and an o-phenylene-diamine (OPD) solution of 1.5 mg/mL of 1,2-phenyldiamine dihydrochloride (DAKO, Glostrup, Denmark) dissolved in water containing 0.015% H₂O₂ and was added to the plates. After approximately 30 min the reaction was stopped by adding 1.25 M H₂SO₄ and the optical density at 492 nm was measured in an ELISA reader.

For each ELISA plate the specificity for a certain SNP/haplotype was examined by comparing the optical density (OD) values of the positive control(s) versus the negative controls (i.e., the controls with a different SNP/haplotype). The OD values of the controls varied between experiments, though only

rarely compromising specificity and as a “rule of thumb” the difference in OD values between positive and negative controls should be > 1.0 . Thus, no fixed threshold value could be specified and for each SNP test a simple analysis of the positive and negative controls to set a threshold for positivity had to be performed for each plate.

The identification of SNPs in *mbl-2* using the SSOP-ELISA method was found to be simple and rapid providing results for the full panel of SNPs in *mbl-2* in 88 samples (i.e., one ELISA plate of samples plus controls) within 1 day. To verify the specificity of the SSOP-ELISA results, the samples were analyzed at polymorphisms at -220 X/Y, $+4$ P/Q, and $52 + 54$ AA/AB by the use of the sequence-specific priming (SSP)-PCR, described by Garred and others.¹⁹ After removal of PCR negatives by the SSOP-ELISA or the SSP-PCR method (negatives in total for analyses of the three polymorphisms, $N = 8$ for both SSOP-ELISA and SSP-PCR out of 106 samples), the two methods gave identical results in 99.0%, 98.1%, and 96.0% of the samples at polymorphisms at -220 X/Y, $+4$ P/Q, and $52 + 54$ AA/AB, respectively (data not shown).

Definition of *mbl-2* haplotype groups. The SNPs of the *mbl-2* gene were combined into MBL haplotypes on the basis of strong linkage disequilibrium between the SNPs resulting in 7 known haplotypes, as described by Garred and others.¹⁹ In brief, four functional MBL haplotypes (LXPA, LYPA, LYQA, and HYPA) and three defective MBL haplotypes (LYPB, LYQC, and HYPD) were grouped as the normal allele “A” and the defective allele “O,” respectively. For statistical analysis purposes, only the X/Y polymorphism at the promoter region (pos. -221) was included. Thus, six MBL haplotype groups were defined according to functional status and promoter status (see Table 3). Individuals with both functional MBL alleles (YA/YA) were further categorized as high-expressing MBL genotypes according to Garred and others,¹⁹ whereas all other individuals were categorized as low-expressing MBL genotypes.

Data analysis. Data were analyzed using the statistical software package SPSS 15.0 (SPSS, Inc., Chicago, IL). The χ^2 analysis was used to compare the communities with regards to distribution of sex, CFA status, and MBL genotype group, whereas Students *t* test was used to compare age distributions. Logistic regression analysis (LRA) was used to analyze the strength of the association between the infection status in 1975 (mf status) and 2006 (CFA status). In addition, LRA was used to determine the association between the MBL genotype group (high or low expressing) and 1) CFA status in 2006 and 2) gain of infection between 1975 and 2006 among those who were mf negative in 1975. The strengths of all associations were calculated as odds ratios (OR) with 95% confidence intervals (CI). All statistical associations were adjusted for potential confounding effects of age, sex, and community.

RESULTS

General characteristics of the study individuals. A total of 104 individuals examined for mf in 1975 were re-identified in 2006 and examined for MBL genotype and CFA (12.8% of all individuals examined in 1975). An overview of these and their infection status in 1975 and 2006 is given in Table 2. There were only minor and statistically non-significant differences between the two communities with regards to sex

TABLE 2

Overview of the study individuals from Tawalani and Kwale and their microfilaria (mf) status in 1975 and circulating filarial antigen (CFA) status in 2006 ($N = 104$)

Village	No. individuals (% of total)	No. males/ females	Mean age in 2006 in years (range)	No. (%) with positive mf status in 1975	No. (%) with positive CFA status in 2006
Tawalani	43 (41.3)	24/19	53.3 (31–88)	13 (30.2)	22 (51.2)
Kwale	61 (58.7)	27/34	52.7 (33–84)	9 (14.8)	30 (49.2)
Total	104 (100.0)	51/53	51.9 (31–88)	22 (21.5)	52 (50.0)

ratio, mean age, and CFA status (χ^2 test and *t* test, $P > 0.05$ for all tests).

Among the re-identified individuals, 21.5% ($N = 22$) and 50.0% ($N = 52$) presented as mf positive in 1975 and CFA positive in 2006, respectively. Among the 82 individuals who were mf negative in 1975, 50 (61.0%) were CFA negative in 2006, whereas among the 22 individuals who were mf positive in 1975, 20 (90.9%) were CFA positive in 2006. Hence, the vast majority of those mf positive in 1975 were still infected in 2006, whereas the majority of those mf negative in 1975 were not infected in 2006. Statistical analysis indicated a very strong and statistically significant association between mf status in 1975 and CFA status in 2006 (OR = 15.63; CI 95%: 3.42–71.42, $P < 0.01$). After adjustment for age, sex, and community this association remained strong and statistically significant (OR = 12.95; CI 95%: 2.65–63.95, $P < 0.01$).

Association between MBL genotype and infection status in 2006. The six MBL genotype groups as defined according to functional status and promoter status, and the distribution of the 104 study individuals in these groups are shown in Table 3. The highest frequency (39%) was found among individuals expressing one functional and one defective MBL allele (YA/O), whereas individuals expressing both functional alleles (YA/YA) accounted for 23% of the examined individuals.

The distributions of high- and low-expressing MBL genotype groups in relation to sex, mean age, and CFA status are shown in Table 4. The majority of the examined individuals had a low-expressing MBL genotype (76.9%). The distribution of low-expressing MBL genotype individuals in Kwale and Tawalani were 72.1% and 80.3%, respectively (χ^2 test, $P > 0.05$). Neither sex distribution nor age differed significantly between the two MBL expression groups (χ^2 and *t* test, $P > 0.05$ for both tests). Among the individuals with a high-versus low-expressing MBL genotype, 29.2% and 56.3% presented with a positive CFA status in 2006, respectively. Hence, low-expressing MBL genotype individuals were three times more likely to present with filarial infection in 2006 compared with high-expressing *mbl-2* genotype individuals (OR = 3.12; CI 95%: 1.17–8.36, $P = 0.035$). After adjusting

TABLE 3

Frequency of MBL genotype polymorphisms in individuals from Tawalani and Kwale villages in Tanga Region, Tanzania*

MBL genotype group	MBL expression	Frequency in sample (number individuals)
YA/YA	High	0.23 (24)
YA/XA	Low	0.19 (20)
XA/XA	Low	0.04 (4)
YA/O	Low	0.39 (41)
XA/O	Low	0.06 (6)
O/O	Low	0.09 (9)

* Categorization of genotype groups are based on promoter and functional status resulting in high and low mannose-binding lectin (MBL) expression.

TABLE 4

Relationship between mannose-binding lectin (MBL) expression group and circulating filarial antigen (CFA) status in 2006

MBL expression group	No. individuals (% of total)	No. individuals in Tawalani/Kwale	No. males/females	Mean age in 2006 in years (range)	No. (%) with positive CFA status in 2006
High	24 (23.1)	12/12	11/13	49.0 (31–78)	7 (29.2)
Low	80 (76.9)	31/49	40/40	54.0 (33–88)	45 (56.3)
Total	104 (100.0)	43/61	51/53	51.9 (31–88)	52 (50.0)

for the effect of sex, age, and community, the statistical association between MBL genotype and CFA decreased slightly but remained statistically significant (OR = 2.90; CI 95%: 1.03–8.36, $P = 0.045$).

Association between MBL expression groups and long-term trends in infection status (1975 versus 2006). A total of 82 individuals were mf negative in 1975. In this group, 50 individuals remained CFA negative (61.0%), whereas 32 individuals (39.0%) were CFA positive in 2006 (Table 5). Among individuals who were infection negative in both surveys or who had gained infection when examined in 2006, 68.0% and 96.9% had a low-expressing MBL genotype, respectively, resulting in a statistically significant association between the *mbi-2* genotype group and the long-term trend in infection status (OR = 14.59; CI 95%: 1.83–116.55, $P = 0.011$). After adjustment for effect of sex, age, and community, the association remained strong and statistically significant (OR = 9.51; CI 95%: 1.14–79.71, $P = 0.038$). Hence, individuals who had a low-expressing MBL genotype were almost 10 times more likely to be infection positive in 2006 than those with a high-expressing MBL genotype.

DISCUSSION

More than 120 million individuals worldwide are estimated to be infected with *W. bancrofti*, and major efforts are currently being made to control the infection through the World Health Organization (WHO) anchored Global Program to Eliminate Lymphatic Filariasis (GPELF).²⁵ There are, however, still many gaps in our understanding of biological aspects of *W. bancrofti* infections, including those related to exposure, host susceptibility, and infection longevity. This study was carried out on a cohort of individuals from two endemic villages in Tanzania, which had been followed regularly for more than three decades. We previously reported that individuals who were infected in 1975 had a much higher risk of being infected in subsequent surveys, suggesting that they were more susceptible to infection and re-infection than other individuals from the same cohort.^{6,7} In agreement with this, the present study indicated that individuals who had infection (microfilaraemia) in 1975 were almost 13 times more likely to present with infection (circulating antigenemia, CFA status) in 2006 than those who

TABLE 5

Relationship between long-term trends in infection status (1975 vs. 2006) and mannose-binding lectin (MBL) expression group among individuals being microfilaria negative in 1975 ($N = 82$)

Infection status in 1975 and 2006*	No. individuals (% of total)	Mean age in 1975 in years (range)	No. with low MBL expression (%)
Negative–negative	50 (61.0)	18.2 (1–70)	34 (68.0)
Negative–positive	32 (39.0)	23.4 (2–55)	31 (96.9)
Total	82 (100.0)	22.6 (1–70)	65 (79.3)

* Microfilarial status in 1975 and circulating filarial antigen status in 2006.

were infection negative in 1975; despite the application of several rounds of mass-treatment in the study area during the intervening period. The study therefore confirmed that the previously observed static infection pattern at the individual level continued to persist for more than three decades. Because the mean lifespan of adult *W. bancrofti* worms in the human host has been estimated to about 10 years,²⁶ the static infection pattern indicates that re-infection commonly occurred in the study population, and that once individuals acquired infection they had a high risk of being re-infected, and thus of remaining infected for long periods—maybe for their entire life.

Studies from Sub-Saharan Africa have previously reported that a high proportion of individuals in this region have low MBL levels, mainly because of a high frequency of the C-variant allele in the *mbi-2* gene allele in the *mbi-2* gene.^{27–30} This was confirmed in this study, in which the vast majority of the examined individuals presented with a low-expression MBL genotype (76.9%), and the defective C-allele was the most common variant (60.0%) (results not shown).

We found a statistically significant association between the MBL genotype group and filarial infection status in 2006, with low MBL expression individuals being 2.9 times more likely to present with infection than high MBL expression individuals. Furthermore, we found a strong association between the MBL genotype group and gain of infection over the three-decade period (OR = 9.5). Taken together, these findings strongly suggest that the risk of infection was associated with the MBL expression genotype, and that the distribution of MBL genotypes in the study cohort contributed to the observed static infection pattern of lymphatic filariasis.

The frequently noted family and household clustering in lymphatic filariasis has generally been ascribed to variation in individuals' exposure to infection,¹⁰ adaptive immune responsiveness,³¹ or genetic composition.¹⁶ There is accumulating evidence that host genetics is an important determinant of infection and morbidity in several helminth infections, including schistosomiasis and ascariasis.³² Associations between *W. bancrofti* infection and genetically determined innate immune responses have previously been assessed in an Indian population, and moderate and strong associations were observed for polymorphisms in the genes coding for a phagocyte-specific chitotriosidase (CHIT1) and MBL, respectively.¹⁷ Recent studies on MBL-deficient mice exposed to mf of *Brugia malayi*—another human filarial parasite—showed that the mf survived for significantly longer time periods in these when compared with wild-type mice.³³ The MBL is known to be an important component of innate immunity toward microbes by activating complement and augmenting opsonization and phagocytosis.^{19,29} Whether MBL deficiency increases susceptibility to filarial infection by preventing these mechanisms, and the role played by MBL in innate immunity to these infections, should be important foci of further studies.

The cohort of individuals used for this study was originally identified and examined three decades ago.⁷ The number of individuals has now been reduced to only a fraction of the original size, as many individuals either died or moved to other places. Hence, the genetic composition of the study cohort may no longer have been entirely representative for the general population in 2006. The cohort had been offered drug treatment against *W. bancrofti* several times during the

three decades. If response to treatment and susceptibility to re-infection are affected by genetic factors, this may to some extent have influenced the distribution of CFA positive individuals in the cohort. Despite these limitations, the results of this study suggest a strong involvement of polymorphisms in the *mb1-2* gene in susceptibility to *W. bancrofti* infection, and this study is—according to our knowledge—the first to show an association between human genetic variation and *W. bancrofti* in a Sub-Saharan African population. The findings indicate a need for further and more detailed studies of the role of human genetic variability in susceptibility to *W. bancrofti* infection and re-infection. Knowledge regarding the importance of specific high-risk groups and how these contribute to the overall transmission dynamics in endemic communities would also be relevant for the large-scale control programs, which are now implemented under the GPELF in many endemic countries worldwide, including countries in the Eastern and Southern African region.

Received June 19, 2009. Accepted for publication October 12, 2009.

Acknowledgments: The inhabitants and village helpers from Kwale and Tawalani are sincerely thanked for their cooperation. We also thank the survey team from the National Institute of Medical Research (NIMR) Filariasis Laboratory in Tanga (Zaina Ali, Charles Guzo) for their dedication to the field work, laboratory technicians Ulla Abildtrup (Centre for Medical Parasitology) and Bente L. Jensen (DBL—Centre for Health Research and Development) for skilled laboratory analyses of the specimens, and Megumi Yamakawa for invaluable assistance during field work.

Financial support: The project received financial support from the Ruth Wiemann Jensen Foundation, Denmark. The development of the MBL PCR-ELISA was partly funded by BioPorto Diagnostics A/S (www.bioporto.com).

Disclosure: The paper is published with the permission of the Director General of NIMR, Tanzania.

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