รายงานวิจัยฉบับสมบูรณ์

การรักษาโรคเท้าช้างแนวทางใหม่โดยการฟื้นฟูภูมิคุ้มกันของโฮสต์

โดย ผศ. ดร. อรภัค เรี่ยมทอง

มิถุนายน 2561

สัญญาเลขที่ TRG5880093

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ผศ. ดร. อรภัค เรี่ยมทอง
ภาควิชาชีวโมเลกุลและพันธุศาสตร์โรคเขตร้อน คณะ
เวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

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Investigator : ผศ. ดร. อรภัค เรี่ยมทอง ภาควิชาชีวโมเลกุลและพันธุศาสตร์โรคเขตร้อน คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล
(ชื่อนักวิจัย)

E-mail Address : onrapak.rea@mahidol.ac.th

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Abstract

In combination, chemotherapy and vaccination should afford effective control of human filariasis. BALB/c mice and gerbils (*Meriones unguiculatis*), two animal disease models, are commonly used to evaluate filarial vaccine candidates. However, the relevance of the information gained from these species to the human immune response has not been investigated. Hence, we used an immunoproteomic approach to identify the *Brugia malayi* immunogens that induce antibody production in microfilaremic humans, infected gerbils, and *B. malayi*-immunized BALB/c mice. Among the 44 proteins in an adult *B. malayi* extract, 11 (heat shock protein 70, intermediate filament protein, Bm9307, aldehyde dehydrogenase, chaperonin-like protein HSP60 family, transglutaminase, independent phosphoglycerate mutase isoform 1, tubulin beta-1 chain, BmFKBP59, enolase and disorganized muscle protein 1) were commonly recognized by the three host species sera. The other 33 proteins showed differential immunogenicity across the species. Therefore, translation of the immunological data obtained from the two rodent species to humans needs careful consideration because immunogenic proteins in the model hosts may not be human immunogens. Several microfilarial-specific antigens formed circulating immune complexes with antibodies from the microfilaremic humans, raising the possibility of this being an immune evasion mechanism used by circulating microfilariae to avoid antibodies, complement attack and antibody-dependent cell-mediated immunity.

Keywords: Animal models of filariasis, *Brugia malayi*, immunome, immune complex, lymphatic filariasis, LC-MS/MS, microfilaremia.

(คำหลัก)

บทคัดย่อ

การควบคุมโรคเท้าช้างเพื่อให้ได้ประสิทธิภาพควรเลือกการผสมผสานทั้งการให้ยาและวัคซีน หนู BALB/c และ gerbil (*Meriones unguiculatis*) เป็นสัตว์ทดลองที่ใช้มาในการศึกษาวัคซีนโรคเท้าช้าง อย่างไรก็ตามไม่เคยมีการศึกษามาก่อนว่าข้อมูลที่ได้จากสัตว์ทั้งสองชนิดนี้จะสอดคล้องกับมนุษย์เพียงใด ดังนั้น การทดลองนี้จึงใช้เทคนิค immunoproteomics เพื่อระบุ immunogens ของพยาธิ *Brugia malayi* โดยใช้แอนติบอดีจากคนไข้ที่มี microfilaria แอนติบอดีจาก gerbil ที่ติดเชื้อ และแอนติบอดีจากหนูที่ถูกกระตุ้นภูมิคุมกันด้วย *B. malayi* lysate จากการทดลองพบว่า 44 immunogens โดยโปรตีน 11 ชนิด ได้แก่ heat shock protein 70, intermediate filament protein, Bm9307, aldehyde dehydrogenase, chaperonin-like protein HSP60 family, transglutaminase, independent phosphoglycerate mutase isoform 1, tubulin beta-1 chain, BmFKBP59, enolase and disorganized muscle protein 1 สามารถทำปฏิกิริยากับแอนติบอดีจากคน หนู BALB/c และ gerbil ได้เหมือนกัน อีก 33 immunogens มีความสามารถทำปฏิกิริยากับแอนติบอดีจากคน หนู BALB/c และ gerbil ได้ต่างกัน ดังนั้น การส่งต่อความรู้จากสัตว์ทดลองไปใช้กับมนุษย์จึงต้องทำด้วยความระมัดระวัง นอกจากนี้แอนติเจนที่อยู่ใน circulating immune complexes จากเลือดของคนถูกระบุชนิดอีกด้วย

คำหลัก: สัตว์ทดลอง โรคเท้าช้าง แมสสเปคโทรเมตรี

Objectives

In this study, an immunoproteomic approach was used to identify and compare the *B. malayi* immunogens that induce antibody production in humans (the definitive host), gerbils (a permissive animal model used commonly for large-scale production of parasites and for studying host immune responses) and mouse (a naturally refractory species). Additionally, we sought to identify the *B. malayi* antigens that were trapped in the circulating immune complexes from the microfilaremic subjects. The information gained from this study should provide useful guidelines of relevance to the current animal models used for studying

immune responses to filarial infections, for filarial vaccine development, and for translating the data gained from animal models to human filariasis.

Specific objectives:

1. To identify and compare the *B. malayi* immunogens that induce antibody production in humans, gerbils and mouse

2. To identify the *B. malayi* antigens that were trapped in the circulating immune complexes from the microfilaremic subjects

Introduction

Lymphatic filariasis (LF) is a human helminthiasis caused by three nematode species: *Wuchereria bancrofti*, *Brugia malayi*, and *B. timori* [1]. The disease has a predilection for warm and humid regions where the intermediate hosts for the nematodes, the mosquito vectors, flourish [2]. *B. malayi* infection is found in South and Southeast Asia including Thailand [3]. Humans get infected through the bites of mosquitoes harboring infective third-stage larvae (L3) in their saliva. In the body, the L3 develop into adult males and females that dwell in the lymphatic system, including the lymph nodes. After mating, the female worms release early stage larvae (microfilariae) into the host's blood circulation causing a microfilaremic state. The circulating microfilariae are taken up with the blood meals of the mosquitoes wherein they develop into infective larvae and can be spread to other hosts. Humans with chronic filarial infections may have an overt pathology called elephantiasis, which involves thickening of the skin and the underlying tissues of the extremities. In addition, lymphoadenoma, chyluria and hydrocele, the latter of which occurs in *W. bancrofti* infections, are caused by occlusion of the lymphatic circulation by adult parasites [4]. Elephantiasis leads to organ deformity and disability. *B. malayi* infection frequently causes tropical pulmonary eosinophilia (TPE), which manifests as a high eosinophil count, mottling appearance of the lungs on chest radiography, fever, paroxysmal cough with sputum, dyspnea, hepatosplenomegaly and weight loss from immune hyper-responsiveness to the filarial worms. People with TPE have lung granulomas, a pseudo-tuberculous condition where the microfilariae are surrounded by eosinophils. Microfilaremia is absent in both elephantiasis and TPE [5].

The World Health Organization (WHO) have estimated that in 2013 about 1.25 billion people in 72 countries and territories were at risk of filarial infections, with 120 million people infected and about 40 million disfigured and incapacitated by the disease [6]. Filariasis is one of the tropical diseases that the WHO Global Programme aims to eradicate and alleviate the physical effects that patients with it experience by 2020 [6]. However, vector control is problematic and mass drug treatment is hampered by non-compliance [7,8], the emergence of drug resistant parasites [9], and the existence of non-human primates that serve as zoonotic reservoirs in the endemic areas. Re-infection and new infections in humans can occur any time; thus, sustained annual mass drug therapy is required, but is difficult to implement [10]. It is envisaged that combined chemotherapy and vaccination should be an effective strategy for controlling filarial infections [11]. Therefore, much current research on filariasis is focused on developing an effective filariasis vaccine and trying to understand the mechanisms underlying the different infection phenotypes in human filarial infections [12]. Many candidate vaccines have been evaluated in modeled animals including BALB/c mice [13,14] and gerbils (Mongolian jirds, *Meriones unguiculatis*) [15,16]. However, how relevant the immune responses of these animal models are to *B. malayi* antigens in the context of human responses awaits investigation.

Thus, in this study, an immunoproteomic approach was used to identify and compare the *B. malayi* immunogens that induce antibody production in humans (the definitive host), gerbils (a permissive animal model used commonly for large-scale production of parasites and for studying host immune responses) [17] and mouse (a naturally refractory species) [18]. Additionally, we sought to identify the *B. malayi* antigens that were trapped in the circulating immune complexes from the microfilaremic subjects. The information gained from this study should provide useful guidelines of relevance to the current animal models used for studying immune responses to filarial infections, for filarial vaccine development, and for translating the data gained from animal models to human filariasis.

Methodology

Ethics statements

The experiments involving gerbils were approved by the Animal Care and Use Committee, Faculty of Medicine, Chiang Mai University, Thailand (No. 05/2558). The mouse experiments received approval from the Animal Care and Use Committee, Faculty of Tropical Medicine, Mahidol University (No. 012-2016). All procedures involving animals

were carried out by scientists who held certificates on "Permission for use of animals in research" of the National Research Council of Thailand (NRCT). Animal husbandry and manipulation conformed to the guidelines of the NRCT. The study protocol was approved by the Institutional Review Board for Human Research, Faculty of Medicine Siriraj Hospital, Mahidol University (No.247/2560; EC2).

Preparation of adult *B. malayi* and worm extracts

For large-scale production of adult *B. malayi*, L3 were injected intraperitoneally into young adult gerbils and adult *B. malayi* worms were harvested from the peritoneal cavities of the infected animals [17]. The worms were washed twice with distilled water and once with 0.01 M phosphate-buffered saline (PBS) pH 7.4 before grinding them in protease inhibitor-containing PBS using a glass tissue grinder. The preparation was sonicated (LABSONICP, France) (30% amplitude, 0.5 cycles, 15 min) on ice and then centrifuged (12000 × g, 4 ^{o}C, 20 min). The supernatant (*B. malayi* extract) was collected and the protein content determined by the Bradford method. The preparation was kept in small aliquots at -20 ^{o}C until use.

Serum Samples

Serum samples were collected from nine human subjects with microfilaremia (designated P1–P9) who resided in the Narathiwat Province of southern Thailand where B. malayi filariasis is endemic. Serum samples from two healthy controls (H1 and H2) living in an area of non-endemic disease served as the negative controls. BALB/c mice (5 weeks old) were purchased from The National Laboratory Animal Center, Mahidol University, Nakhon Pathom Province, Thailand. Two mice were each injected intraperitoneally with 40 μg of the B. malayi extract mixed with alum adjuvant (Pierce, Waltham, MA, USA). Four booster doses (80 μg/dose) of the same immunogen by the same route were given to the primed mice at two-weekly intervals. Mice were bled on day 7 after the last booster and their serum samples (designated MI1 and MI2) were collected separately. Serum samples from two sham-treated mice injected intraperitoneally with PBS (MS1 and MS2) were similarly prepared for use as the negative controls. The serum samples from two infected gerbils (designated GI1 and GI2) were collected on the day that they were sacrificed for adult *B. malayi* collection. Sera from two uninfected gerbils (GU1 and GU2) were also prepared.

Two-dimensional gel electrophoresis (2DE) and 2DE immunoblotting

The *B. malayi* extract was cleaned using a 2D-cleanup kit (GE Healthcare Bioscience, UK) to eliminate any non-protein contaminants. The preparation (75 µg) was applied to individual IPG strips (3–10 isoelectric point, pI; GE Healthcare) and placed on a strip holder (Ettan IPG Phor Electrofocusing System; GE Healthcare). PlusOne DryStrip Cover Fluid (GE Healthcare) was overlaid on the IPG strips and the preparations were incubated at 20 ºC for 12 h. The first dimension electrophoresis was performed at 300 V for 30 min, 1000 V for 30 min, and 5000 V for 72 min, and the focused IPG strips were treated with 10 mg/mL dithiothreitol (DTT) at 25 ºC for 15 min and 25 mg/mL iodoacetamic acid (IAA) for 15 min. The second dimension electrophoresis was conducted by placing each focused IPG strip onto a 12% SDS gel cast in the Hoefer SE 260 system (Amersham Bioscience, UK). Electrophoresis was performed at 75 V per gel until the blue dye reached to the lower gel edge. Protein spots on the gel were visualized by Coomassie Brilliant Blue G-250 dye (CBB) staining (BioRad, USA).

For the 2DE immunoblotting, the proteins on the 2DE gels were transblotted onto nitrocellulose membranes (Merck Millipore, Ireland) using the Amersham TE 70 semi-dry transfer unit (GE Healthcare, USA). The blotted membranes were blocked with 5% skimmed milk in Tris buffered-saline containing 0.1% Tween-20 (TBS-T) before placing in solutions of individual human sera (P1–P9, H1 and H2), mouse sera (MI1, MI2, MS1 and MS2) or gerbil sera (GI1, GI2, GU1 and GU2) each diluted 1:20 in TBS-T and kept at 4 ºC overnight. After washing, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated anti-isotype antibody (KPL, USA) at a 1:2500 dilution for 1 h. LuminataTM Classico Western HRP substrate (Millipore Corporation, USA) was used to visualize the antigen–antibody reactive spots under the ImageQuant LAS 4010 (GE Healthcare). The protein spots on the CBB-stained 2DE gels that matched the antibody-reactive spots on the respective immunoblot membranes, but did not react with the negative control sera, were excised and then digested with trypsin for protein identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Identifying the *B. malayi* antigens that formed immune complexes in the blood circulation of microfilaremic subjects

PureProteome™ Kappa Ig Binder Magnetic Beads (LSKMAGKP02 kit; Millipore Corporation, USA) were used to capture the immune complexes in the sera from the subjects with microfilaria. The beads (300 µL) were pipetted into two microfuge tubes and then placed in a magnetic stand and the bead storage buffer was removed. The magnetic beads

were washed twice in 500 µL of binding buffer from the kit. Pools of P1–P9 sera (5 µL each) diluted in binding buffer to 100 µL volumes were added and mixed with the magnetic beads in a single tube and the pool of H1 and H2 sera were added to the beads in a different tube. Both tubes were rotated at room temperature for 60 min before their placement in the magnetic stand, and the immune complex-depleted fluids were discarded. The beads were washed thoroughly in binding buffer, and the immune complexes were eluted by suspending the beads in 150–200 µL of elution buffer from the kit with end-over-end rotated for 5–10 min. The eluates from both tubes were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by CBB staining. The gels were cut horizontally into equal pieces, subjected to in-gel trypsin digestion and the *B. malayi* proteins were identified by LC-MS/MS.

In-gel protein digestion

Gel pieces containing the CBB-stained proteins were destained by adding acetonitrile (50%) in 50 mM NH_4HCO_3 until they were colorless. Each gel piece was placed in 10 mM DTT at 60 ^oC for 15 min (reduction) and in 55 mM IAA in 50 mM NH_4HCO_3 at room temperature in darkness for 30 min (alkylation). Thereafter, all the gel pieces were dehydrated with 100% acetonitrile (ACN) (Sigma-Aldrich, USA) and kept at room temperature. Protein digestion was performed at 37 ^oC overnight by adding 0.1 mg/mL of trypsin (Sigma-Aldrich) in 50 mM ammonium bicarbonate to each gel piece. The peptides were extracted by adding ACN at 50% final concentration. Each supernatant was transferred to a new microfuge tube and all the solutions were dried in a centrifugal concentrator (TOMY, Japan) at 45 ^oC.

LC–MS/MS analysis

Each tryptic-digested preparation was resuspended in 0.1% of formic acid and then subjected to liquid chromatography (LC) (Ultimate 3000 nano-LC system; Dionex, UK). LC was performed at a 300 nL per min flow rate under a 45 min gradient using the Acclaim PepMap RSLC 75 µm × 15 cm nanoviper C18, 2 µm particle size, 100 Å pore size (Thermo Scientific, Waltham, MA). Mobile phase A was 2% (v/v) ACN and 0.1% (v/v) formic acid in HPLC-grade water and mobile phase B was 0.1% (v/v) formic acid in ACN. The separated peptides were sprayed onto the MicroToF Q II mass spectrometer (Bruker, Bremen, Germany) controlled by Hystar software (Bruker Daltonics, Germany). The MS spectra covered a mass range of m/z 400–2500 and the MS/MS spectra covered a mass range of m/z 50–1500.

The raw MS files were processed to .mgf files by Compass DataAnalysis software (Bruker Daltonics) and thereafter searched by the in-house Mascot server (version 2.3.0; Matrix Science, USA). The trypsin was set as the enzyme and only one missed cleavage site was allowed. The variable modifications selected were carbamidomethyl (C) and oxidation (M). The National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/) was set as the database. MS peptide and MS/MS tolerances were set at 0.6 Da and 0.8 Da, respectively. False-positive identification was reduced by only reporting the peptides with 95% confidence levels.

Protein function prediction

Protein domains were predicted according to their patterns and motifs. The PROSITE database (released on 20-Dec-2017 and containing 1797 documentation entries, 1309 patterns, 1202 profiles and 1223 ProRule) was used [19]. Pfam version 31.0 (March 2017, 16712 entries) was also used for protein function prediction [20].

Results

Immunomes from adult *B. malayi* extracts were identified using serum samples from humans, gerbils and mice

The adult *B. malayi* parasite extract after 2DE separation and protein staining revealed protein spots that were abundant in the 26–95 kDa range, with a pI of 5–9 (Fig. 1).

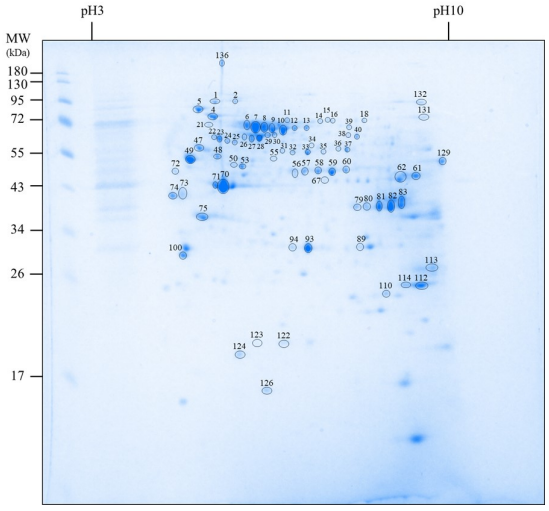

Fig. 1. Two-DE separation pattern of proteins from *B. malayi* adult worms. The worm extract was separated using IPG strip (pI 3–10) and 12% SDS-PAGE. The separated proteins were stained with CBB G-250 dye. Numbers on the left are protein molecular masses (kDa). Proteins in numbered circles were identified by LC-MS/MS.

The proteins in the 2DE gels were blotted onto nitrocellulose membranes and probed individually with the sera from microfilaremic (P1–P9) and healthy subjects (H1 and H2), infected (GI1 and GI2) and non-infected (GU1 and GU2) gerbils, and immune (MI1 and MI2) and non-immune mice (MS1 and MS2). None of the control sera from the three species reacted with the *B. malayi* proteins on the blots (data not shown). Examples of these immunoblots are shown in Fig. 2.

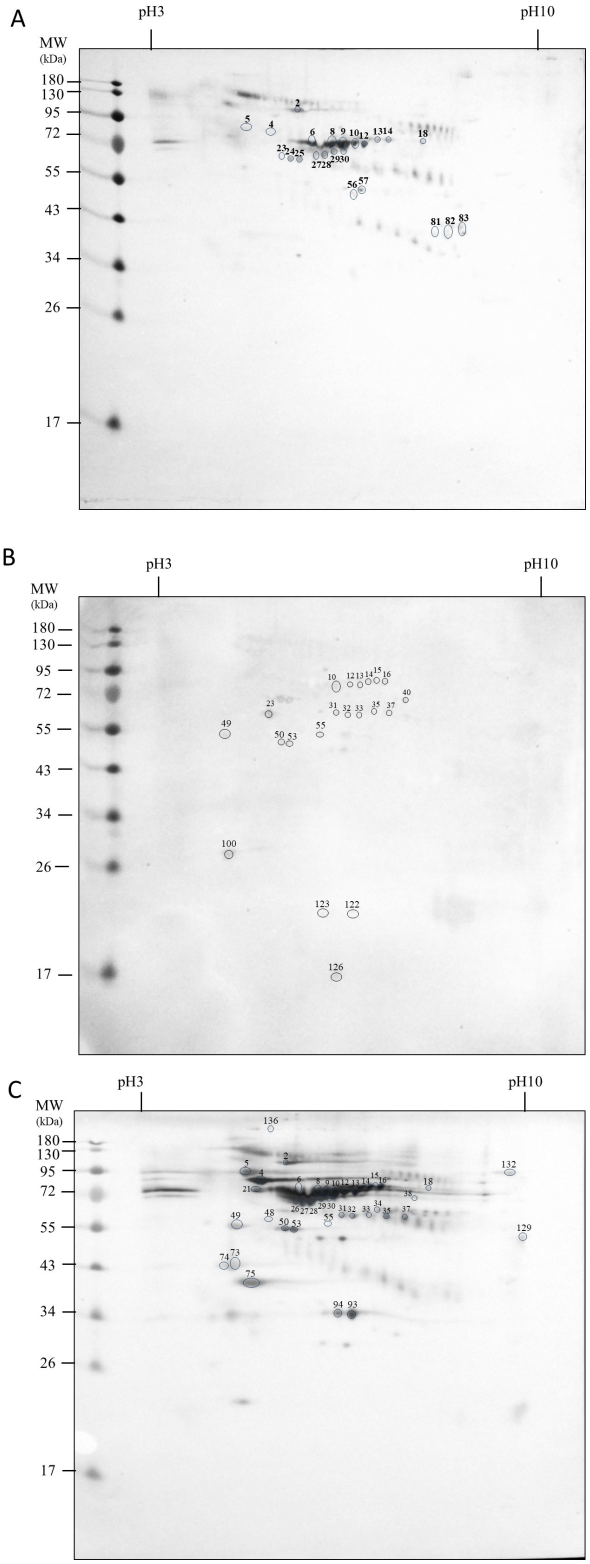

Fig. 2. *B. malayi* antigen patterns revealed by immunoblotting with sera from (A) microfilaremic humans, (B) infected gerbils and (C) immunized mice.

From the 2DE immunoblotting experiment, 94, 56, and 130 spots were captured by immune sera from the nine microfilaremic subjects, two infected gerbils and two immunized mice, respectively. However, only 132 CBB stained-spots corresponding to the immunomic spots were present on the 2DE-gel. Proteins from these 132 spots on the CBB-stained 2DE gel were subjected to LC-MS/MS, and a total of 44 proteins were identifiable. Among them, 34, 20 and 35 proteins were reactive with the sera from the microfilaremic humans, infected gerbils and immunized mice, respectively. Table 1 shows the 44 proteins identified in the 132 immunoreactive spots.

Table 1 *B. malayi* antigens that reacted with antibodies in sera of microfilaremic humans, *B. malayi* infected gerbils and mice immunized with *B. malayi* extract

Spot no.	Accession no.	Protein	Human (n=9)	Gerbil (n=2)	Mouse (n=2)
1	gi|170589273	Endoplasmin precursor	3*	0	0
2	gi|170595872	Transitional endoplasmic reticulum ATPase TER94	4	0	2
4	gi|170574862	Heat shock 70 kDa protein C precursor	**3**	**1**	**2**
5	gi|3096951	Heat shock protein 90	3	0	2
6, 7, 8	gi|170590876	Heat shock 70 kDa protein	9	0	2
9, 26, 29, 30	gi|170590852	Cytoplasmic intermediate filament protein	9	0	2
10	gi|170591494	Intermediate filament protein	**6**	**1**	**2**
12, 13	gi|909114256	Bm9307	**5**	**1**	**2**
14, 15	gi|170584665	Aldehyde dehydrogenase	**1**	**2**	**2**
16	gi|170593397	Moesin/ezrin/radixin homolog 1	0	2	2
18	gi|170583255	Phosphoenolpyruvate carboxykinase [GTP]	3	0	2
21	gi|170593817	Vacuolar ATP synthase catalytic subunit A	1	0	2
22, 23	gi|262092496	Chaperonin-like protein HSP60	**4**	**2**	**1**
24, 25	gi|58418924	Chaperonin GroEL (HSP60 family)	2	0	1
27,28	gi|170596673	Cytoplasmic intermediate filament protein	7	0	2
31	gi|170577077	Vacuolar ATP synthase subunit B	0	1	2
32, 33	gi|170584907	Transglutaminase	**2**	**1**	**2**

34, 35, 37	gi|37681504	Independent phosphoglycerate mutase isoform 1	**1**	**1**	**2**
36	gi|170586616	Gelsolin	1	0	1
38, 40	gi|170587734	Pyruvate kinase, M2 isozyme	0	2	2
39	gi|170576173	Transketolase	0	1	1
47	gi|170588855	Tubulin alpha chain	3	1	0
48	gi|170591793	ATP synthase beta chain, mitochondrial precursor	0	0	2
49	gi|135445	Tubulin beta-1 chain	**4**	**1**	**2**
50, 53	gi|170596615	FKBP-type peptidyl-prolyl cis-trans isomerase-59, BmFKBP59	**3**	**1**	**2**
55-60	gi|170582775	Enolase	**4**	**1**	**2**
61, 62	gi|170593053	Calponin homolog OV9M	2	0	1
67, 69, 70, 136	gi|170581695	Actin 1	3	0	1
71	gi|170579411	Actin	1	0	0
73	gi|170571921	Tropomyosin family protein	1	0	2
74	gi|170595905	EF hand family protein	1	0	2
75	gi|170589519	Disorganized muscle protein 1	**5**	**1**	**2**
79- 82	gi|170590178	Glyceraldehyde 3-phosphate dehydrogenase	7	0	1
83	gi|170578753	Fructose-bisphosphate aldolase 1	1	0	0
89	gi|170592110	Galectin	1	0	1
93, 94	gi|170583137	Galectin	3	0	2
100	gi|584596189	14-3-3 zeta	3	2	0
110	gi|170587487	Immunoglobulin I-set domain containing protein	0	0	1
122	gi|870911	Small heat shock protein	0	1	0

123	gi|170596993	Eukaryotic translation initiation factor 5A-2	0	1	0
124	gi|21436483	Thioredoxin	1	0	0
126	gi|1518125	Small heat shock protein	0	1	0
129	gi|170584161	Elongation factor 1-alpha (EF-1-alpha)	4	0	1
132	gi|170579986	Hypothetical protein Bm1_18045	0	0	1

*, frequency of binding among the tested serum samples

Bold numbers, proteins that were bound by antibodies of all three host species

The protein immunogenicity, in terms of the binding frequencies of the serum antibodies from the infected humans, gerbils and immunized mice, is also shown in Table 1. A Venn diagram showing the number of *B. malayi* antigens that were bound or not bound by the sera from the three host species is shown in Fig. 3.

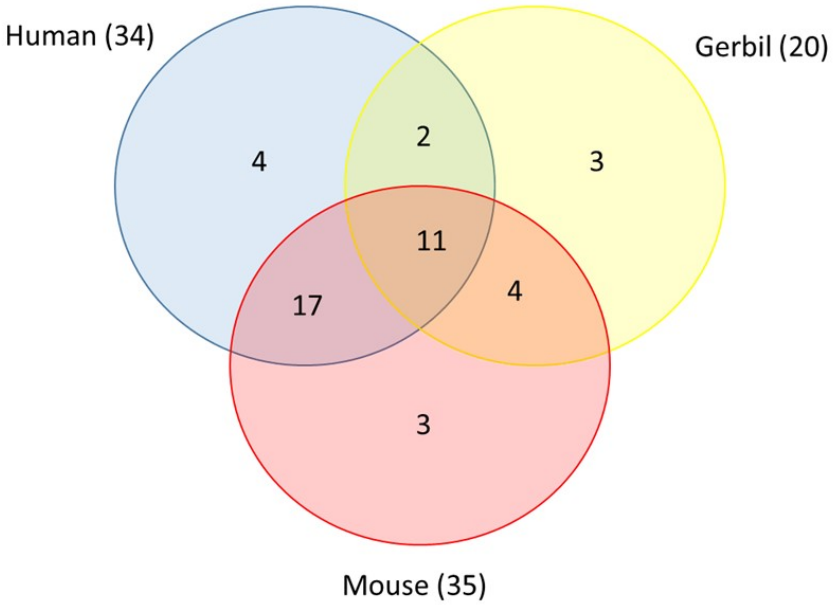

Fig. 3. Venn diagram illustrating the *B. malayi* antigens identified from 2DE-immunoblotting. Antigens reacting with the sera from nine microfilaremic humans (blue), two infected gerbils (yellow), and two immunized BALB/c mice (pink).

Eleven *B. malayi* proteins commonly reacted with the sera from all three species (bold numbers, Table 1). The 11 protein comprised the following: heat shock protein (HSP) 70 (spot 4), intermediate filament protein (spot 10), Bm9307 (spots 12,13), aldehyde dehydrogenase (spots 14,15), chaperonin-like protein HSP60 family (spots 22,23), transglutaminase (spots 32,33), independent phosphoglycerate mutase isoform 1 (spots 34,35,37), tubulin beta-1 chain (spot 49), FKBP-type peptidyl-prolyl cis-trans isomerase-59 or BmFKBP59 (spots 50,53), enolase (spots 55-60), and disorganized muscle protein 1 (spot 75).

All nine serum samples from microfilaremic subjects reacted with the heat shock 70 kDa protein (spots 6–8) and the cytoplasmic intermediate filament protein (gi 170590852; spots 9,26,29,30) (Table 1).

Among the 34 *B. malayi* proteins that induced immune responses in the infected humans, six of them (i.e., endoplasmin precursor, spot 1; tubulin alpha chain, spot 47; actin, spot 71; fructose biphosphate aldolase 1, spot 83; 14-3-3 zeta, spot 100; and thioredoxin, spot 124), were not recognized by the immune sera from the two BALB/c mice.

Sera from the infected mice recognized 35 *B. malayi* proteins. However, seven of these *B. malayi* proteins (moesin/ezrin/radixin homolog 1, spot 16; vacuolar ATP synthase subunit B, spot 31; pyruvate kinase M2 isozyme, spots 38 and 40; transketolase, spot 39; ATP synthase beta chain mitochondrial precursor, spot 48; immunoglobulin I-set domain containing protein, spot 110; and hypothetical protein Bm1_18045, spot 132) were not immunogenic in the infected humans.

Of the 20 *B. malayi* proteins bound by the antibodies from the infected gerbils in the 2DE immunoblotting, seven of them (moesin/ezrin/radixin homolog 1, spot 16; vacular ATP synthase subunit B, spot 31; pyruvate kinase M2 isozyme, spots 38 and 40; transketolase, spot 39; small heat shock protein gi870911, spot 122; eukaryotic translation initiation factor 5A-2, spot 123; and small heat shock protein gi1518125, spot 126) were not immunogenic in nine human patients.

B. malayi **proteins that formed circulating immune complexes with human antibodies** ***in vivo***

The circulating immune complexes captured from the pooled sera from healthy controls and microfilaremic subjects using human immunoglobulin binder magnetic beads were subjected to SDS-PAGE and protein staining. No *B. malayi* proteins were found in the

preparation from the healthy serum pool (data not shown). The gel containing the *B. malayi* proteins that formed immune complexes with the serum pool from the *B. malayi*-infected humans was divided horizontally into 12 pieces (Fig. 4).

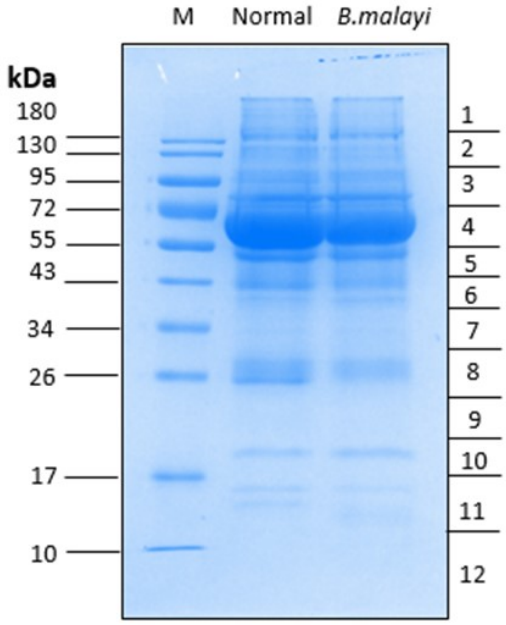

Fig. 4. Enriched circulating antigens identified by 12% SDS-PAGE analysis of negative control sera from healthy subjects and pooled sera from microfilaremic subjects The gel was cut into 12 pieces for protein identification. The protein standard marker (10–180 kDa range) is indicated on the left hand side.

The proteins in the individual gel pieces were identified and seven *B. malayi* antigens (i.e., hypothetical protein Bm1_12480, pao retrotransposon peptidase family protein, uncoordinated protein 44, UDP-glucose/GDP-mannose dehydrogenase family, which contains the NAD binding domain, C2 domain containing protein, FLJ90013 protein, and chaperonin-like protein HSP60) were identified (Table 2).

Table 2 *B. malayi* proteins in immune complexes derived from pooled sera of microfilaremic subjects

No.	Accession no.	Protein name	Score	M.W. (Da)	E-value	Sequence
1	gi|170577330	Hypothetical protein Bm1_12480	68	27732	0.012	NFCDVSICK
					0.16	VIAQSLVCHLFISR
2	gi|170581346	Pao retrotransposon peptidase family protein	110	204026	0.2	WIALFTCFTTR
					0.41	YQVAWPWKDNSSK
					0.17	FLWVKDIQK
3	gi|170588307	Uncoordinated protein 44, isoform e	72	208434	0.017	VNVAEELVK
					0.023	NQMDIASTLLHYR
4	gi|170592475	UDP-glucose/GDP-mannose dehydrogenase family, NAD binding domain containing protein	126	50833	0.02	ESSAIHIVK
					0.11	AIAEYSCGPK
					0.07	NAQLIFMSVNTPTK
5	gi|170593515	C2 domain containing protein	63	38868	0.03	KLYVTVSK
					0.14	MLQFTVYNFVR
6	gi|170593563	FLJ90013 protein	97	68683	0.017	IPIELEK
					0.09	AFSDYSDQVSR
					0.12	TKSLAIFK
7	gi|262092496	Chaperonin-like protein HSP60	69	61217	0.0314	GKPEDLEK
					0.12	AGIIDPTK

E-value, chance of being falsely defined

Among the seven proteins, chaperonin-like protein HSP60 was the only *B. malayi* antigen that was detected by both 2DE-immunomics and immune complex analysis. Bioactivities of the *B. malayi* proteins that could induce antibody production in microfiraremic humans are shown in Table 2.

The characteristics and domain functions of the *B. malayi* proteins that formed immune complexes with the human antibodies

The characteristics and functional domains of the six *B. malayi* proteins that were found only in the immune complexes with the human antibodies were predicted using Prosite and Pfam bioinformatic tools and the results are shown in Tables 3. The six proteins were

predicted as hypothetical protein Bm1_12480, Pao retrotransposon peptidase family protein, isoform e of uncoordinated protein 44, NAD binding domain containing protein of the UDP-glucose/GDP-mannose dehydrogenase family (which contained ankyrin repeat region, ZU5 domain with C-terminal death domain), C2 domain containing protein, and FLJ90013 protein of the eukaryotic membrane protein family.

Table 3. Bioactivities of the *B. malayi* proteins that could induce antibody production in microfiraremic humans

Function	Protein name
Structural protein	intermediate filament protein, moesin/ezrin/radixin homolog 1, gelsolin, tubulin alpha chain, tubulin beta-1, calponin homolog OV9M, actin, tropomyosin family protein, disorganized muscle protein 1, uncoordinated protein 44, C2 domain containing protein
Signaling protein	phosphoenolpyruvate carboxykinase, vacuolar ATP synthase catalytic subunit A, osteoclast isoform, vacuolar ATP synthase subunit B, pyruvate kinase
Protein folding	endoplasmin precursor, heat shock 70 kDa protein C, heat shock protein 90, heat shock 70 kDa protein, chaperonin-like protein HSP60, chaperonin GroEL, transglutaminase, FKBP-type peptidyl-prolyl cis-trans isomerase-59, small heat shock protein, 14-3-3 zeta
Catalytic activity	aldehyde dehydrogenase, independent phosphoglycerate mutase isoform 1, ATP synthase beta chain, enolase, glyceraldehyde 3-phosphate dehydrogenase, fructose-bisphosphate, aldolase 1, Pao retrotransposon peptidase, UDP-glucose/GDP-mannose dehydrogenase family, NAD binding domain containing protein
Protein degradation	transitional endoplasmic reticulum ATPase TER94
Gene regulation	eukaryotic translation initiation factor 5A-2, elongation factor 1-alpha
Antioxidant	thioredoxin
Cell adhesion	immunoglobulin I-set domain, galectin
Binding	EF hand family protein
Hypothetical protein	Bm9307, hypothetical protein Bm1_18045, hypothetical protein Bm1_12480, FLJ90013 protein

Black, Proteins that were found in serum samples of the microfilaremic humans

Red, Proteins that were found only in immune complexes of microfiaremic humans

Blue, Protein that was found in both serum samples and immune complexes

Discussion

Several vaccine candidates for LF have been screened by a variety of techniques and the selected candidates evaluated for protective immunity in laboratory animals (mainly inbred mice) with varying protective results [11-18,21-23]. Proteomes, transcriptomes and secretomes in filarial parasites have been studied [24-32], as have the cellular immune responses in gerbils during experimental filarial infections [33,34]. However, comparative data on the parasite antigens that induce antibody responses in different host species, including humans as the definitive filarial host, gerbils as the permissive host and mice as the naturally refractory host, are lacking. Thus, in this study, we conducted an immunomic analysis to reveal the *B. malayi* antigens that induce antibody responses in microfilaremic humans (who lack tissue pathology), in gerbils that harbored adult parasites, and in mice immunized with *B. malayi* extract.

Only 132 CBB stained-spots corresponding to the immunomic spots recognized by microfilaremic sera (94 spots), infected gerbils (36 spots) and immunized mice (130 spots) were present on the 2DE-gel, indicating that the other antigenic components that were absent in the CBB-stained-2DE gel might not be proteins (because they were not stained by CBB), or they might be proteins that were present in minute amounts in the worm extract and therefore not revealed by the gel-based proteomic approach. It is known that 2DE is fairly insensitive to proteins of low abundance and cannot be used for complete proteomic searches [35]. Therefore, the antigenic components missing in the immunomic spots from the CBB-stained gel were not identified.

Among these 11 proteins that were commonly recognized by sera of the three host species, *B. malayi* transglutaminase has been tested as a DNA vaccine for LF [36]. Mice immunized intramuscularly with this protein each had a marked Th1 response, based on antibody isotype, cytokine profile and splenocyte proliferation upon antigenic stimulation [36]. The independent phosphoglycerate mutase is a highly conserved enzyme involved in glycolysis and gluconeogenesis (interconversion of 3-phosphoglycerate and 2-phosphoglycerate) in filarial worms. The *W. bancrofti* independent phosphoglycerate mutase isoform-1 gene shares 99.4 and 96.0% amino acid sequence identity with *B. malayi* and *Onchocerca volvulus*, respectively. Serine and 13 other amino acid residues involved in the catalytic function of this enzyme are highly conserved [37]. This protein has not been tested as a vaccine candidate, but it is a potential drug and diagnostic target because of its conserved function and pivotal activity in filarial parasites. The protein can also be used as a taxonomical and evolutionary marker for filarial worms [37]. Enolase has been previously identified in excretory–secretory products from *B. malayi* [32]. Its detection by serum IgG1 from asymptomatic subjects from endemic regions, subjects with lymphangitis, and serum IgG4 from microfilaremics evidences its immunogenicity [26]. The role of the remaining eight proteins as vaccine and diagnostic targets and/or drug targets awaits investigation. The data gained from the experimental mice and gerbils on these proteins should be pertinent to humans.

Serum samples from all nine microfilaremic subjects reacted with the heat shock 70 kDa protein and the cytoplasmic intermediate filament protein indicating that the antibodies to these proteins have potential as reliable and accurate epidemiological markers for *B. malayi* exposure. Both proteins were immunogenic in each of the *B. malayi*-immunized BALB/c mice. Previous studies have shown that immunization of BALB/c mice with alum-precipitated recombinant *B. malayi* heat shock protein 70 conferred partial protection against

subsequent challenge with *Litomosoides sigmodontis,* the rodent filarial parasite [38]. Moreover, the heat shock 70 kDa protein was identified as an immunoreactive protein in *B. malayi*, as IgM, IgG1 and IgG4 antibodies to this protein were detected in the sera from patients with different spectrums of clinical filariasis [26]. The cytoplasmic intermediate filament protein was also bound by sera from the two immunized BALB/c mice. Thus, the inbred BALB/c mouse strain should be a suitable model for human filariasis in studies on immune responses against the two *B. malayi* proteins. The heat shock protein 70 was immunogenic in one of the two infected gerbils, unlike the cytoplasmic intermediate filament protein, indicating that gerbils should not be used for immunological studies on these two *B. malayi* proteins. The intermediate filament protein (gi 170591494, spot 10) is an excretory–secretory product of *B. malayi* adult worms [39]. Immunization of *Mastomys coucha*, a rodent genus (family Muridae) endemic to Africa, and gerbils with this protein induced Th1/Th2 type responses that protected the hosts from parasite challenge, indicating that it might be a promising vaccine candidate [40]. However, careful translation of the data obtained from the two outbred rodent species to human is still needed.

B. malayi endoplasmin precursor, tubulin alpha chain, actin, fructose biphosphate aldolase 1, 14-3-3 zeta, and thioredoxin, that were bound by human immune sera were not recognized by the immunized BALB/c mice, indicating that studies on immune response to these proteins should not be conducted with the BALB/c mice. On contrary, moesin/ezrin/radixin homolog 1, vacuolar ATP synthase subunit B, pyruvate kinase M2 isozyme, transketolase, ATP synthase beta chain mitochondrial precursor, immunoglobulin I-set domain containing protein, and hypothetical protein Bm1_18045 that were immunogenic in the BALB/c mice were not immunogenic for humans. Thus, any research results based on these proteins in mice will not be applicable to human infections. Likewise, moesin/ezrin/radixin homolog 1, vacular ATP synthase subunit B, pyruvate kinase M2

isozyme, transketolase, small heat shock protein gi870911, eukaryotic translation initiation factor 5A-2, and small heat shock protein gi1518125 were immunogenic for the infected gerbils but were not bound by the infected human sera. Thus, any research results based on these proteins in gerbils will not be applicable to human infections.

Among the seven *B. malayi* proteins found in circulating immune complexes of the microfilaremic humans, only the chaperonin-like protein HSP60 was detected by both 2DE-immunomics and immune complex analysis, indicating that antibodies against it are partly involved in immune complex formation. The hypothetical protein Bm1_12480 was predicted to contain a mini-chromosome maintenance (MCM) replisome factor domain (an MCM binding domain), which is responsible for DNA replication licensing in eukaryotic cells. This protein has been found to bind to the origin of replication to unwind the double-stranded DNA for DNA synthesis initiation [41]. The Pao retrotransposon peptidase comprises three important domains: reverse transcriptase, Pao retrotransposon peptidase, and integrase. This retrotransposon is capable of self-amplification in a genome. The DNA sequence is first transcribed into RNA, then converted back into an identical DNA sequence by reverse transcription, after which the DNA is integrated back into the genome [42]. This activity can generate phenotypic diversity [43] and facilitate DNA repair [44]. It has also been suggested that Pao is involved in parasite lifestyle adaptation [45]. The uncoordinated protein 44 sequence comprises an ankyrin repeat region, and ZU5 and death domains. The death domain is the cytoplasmic portion of the tumor necrosis factor (TNF) receptor and is involved in TNF-mediated cell death signaling [46]. UDP-glucose/GDP-mannose dehydrogenase generally converts an alcohol to an acid and produces intermediate substances for carbohydrate synthesis [47]. In *Caenorhabditis elegans*, mutation of the gene encoding this enzyme causes defects in female sex organ morphogenesis during the L4 stage [48]. The functions of the C2 domain-containing protein and the FLJ90013 protein in parasites are

elusive, but they are possibly associated with the parasite cell surface [49,50]. These six proteins in the immune complexes were not identified in immunomics of adult *B. malayi* extract using human sera (IgM, IgG1 and IgG4) with various clinical entities of filariasis, including endemic asymptomatic, lymphangitis, elephantiasis, and microfilaremia [26]. Also, they were not detected in proteomics of the adult *B. malayi* extract [our unpublished data], indicating that the six proteins are microfilaria-specific. Free antibodies to the six proteins were not identified in sera from the microfilaremics. The antigen–antibody immune complexes that are formed might be a mechanism used by the circulating microfilaria to avoid antibodies, complement attack and antibody-dependent cell-mediated immunity. Antigen shedding, immune complex formation and surface antigen reduction are strategies used by several parasites to evade host immunity [51,52].

Conclusions

By using a gel based-immunoproteomic approach, this study generated information on pools of *B. malayi* immunogens that reacted commonly and uniquely with antibodies in the sera from microfilaremic humans and two animal models of human filarial infection (i.e., immunized inbred BALB/c mice and infected outbred gerbils). Based on the comparative immunomic profiles of the three species, the suitability of the models and their relevance for translating the immunological data gained from the experimental non-human animals to humans was discovered. Of particular note, the microfilaria-specific antigens that had formed circulating immune complexes in the microfilaremic sera from humans were also identified. Continuous mopping-up of host antibodies by these parasite antigens would help to minimize the detrimental effects of the host antibodies in mediating immune attack on the worms or interfering with the activities of the worms, thereby allowing the microfilaria to survive in the definitive host for onwards transmission.

References

[1] M.J. Taylor, A. Hoerauf, M. Bockarie. Lymphatic filariasis and onchocerciasis. Lancet 376 (9747), (2010) 1175–1185.

[2] J.F.B. Edeson, T. Wilson. The epidemiology of filariasis due to *Wuchereria Bancrofti* and *Brugia malayi*. Annu. Rev. Entomol. 9 (1) (1964) 245–268.

[3] I. Tada. Pathogenesis and treatment of chronic symptoms with emphasis on chyluria and elephantiasis. Trop. Med. Health 39 (1 Suppl 2) (2011) 47–50.

[4] A.W. Chowdhury, C.Z. Amitavsaha, A.S. Abdullah. Occult filariasis infection: could it be a cause of chronic dry cough? J. Med. 10 (1) (2009) 3–6.

[5] World Health Organization. Global programme to eliminate lymphatic filariasis: progress report, 2016. Wkly. Epidemiol. Rec. 40 (2017) 598–608.

[6] M. El-Setouhy, K.M. Abd Elaziz, H. Helmy, H.A. Farid, H.A. Kamal, R.M. Ramzy. The effect of compliance on the impact of mass drug administration for elimination of lymphatic filariasis in Egypt. Am. J. Trop. Med. Hyg. 77 (6) (2007) 1069–1073.

[7] B.V. Babu, S. Mishra. Mass drug administration under the programme to eliminate lymphatic filariasis in Orissa, India: a mixed-methods study to identify factors associated with compliance and non-compliance. Trans. Roy. Soc. Trop. Med. Hyg. 102 (12) (2008) 1207–1213.

[8] A.E. Schwab, T.S. Churcher, A.J. Schwab, M.G. Basanez, R.K. Prichard. An analysis of the population genetics of potential drug-resistance in *Wuchereria bancrofti* due to combination chemotherapy. Parasitology 134 (pt 7) 2007) 1025–1040.

[9] J. Horton. The development of albendazole for lymphatic filariasis. Ann Trop Med Parasitol. 3 (Suppl 1) (2009) S33–S40.

[10] A. Samykutty, G. Dakshinamoorthy, R. Kalyanasundaram. Multivalent vaccines for lymphatic filariasis. Procedia Vaccinol. 3 (2010) 12–18.

[11] D. Abraham, R.B. Grieve, J.M. Holy, B.M. Christensen. Immunity to larval *Brugia malayi* in BALB/c mice: protective immunity and inhibition of larval development. Am J Trop. Med. Hyg. 40 (6) (1989) 598–604.

[12] A. Kwarteng, S.T. Ahuno. Immunity in filarial infections: lessons from animal models and human studies. Scan. J. Immunol. 85 (4) (2017) 251-257.

[13] B.W. Li, A. Rush, S.R. Zhang, K.C. Curtis, G.L. Weil. Antibody responses to *Brugia malayi* antigens induced by DNA vaccination. Filarial J. 3 (1) (2004) 1

[14] B.W. Li, R. Chandrashekar, G.J. Weil. Vaccination with recombinant filarial paramyosin induces partial immunity in *Brugia malayi* infection in jirds. J. Immunol. 150 (5) (1993) 1881–1885.

[15] S. Thirugnanam, P. Pandiaraja, K. Ramaswamy, V. Murugan, M. Gananaseker, K. Nandakumar. *Brugia malayi*: comparison of protective immune responses induced by BM-alt-2 DNA, recombinant BM-ALT-2 protein and prime-boost vaccine regimens in a jird model. Exp. Parasitol. 116 (4) (2007) 483–491.

[16] A. Saeung, W. Choochote. Development of a facile system for mass production of *Brugia malayi* in a small-space laboratory. Parasitol. Res. 112 (9) (2013) 3259–3265.

[17] E. Pearlman, W.K. Kroeze, F.E.J. Hazlett, S.S. Chen, S.D. Mawhorter, W.H. Boom, J.W. Kazura. *Brugia malayi*: acquired resistance to microfilariae in BALB/c mice correlates with local Th2 responses. Exp. Parasitol. 76 (2) (1993) 200–208.

[18] A. Dfaz, J.E. Allen. Mapping immune response profiles: the emerging scenario from helminth immunology. Eur. J. Immunol. 37 (12) (2007) 5319–5326.

[19] C.J.A. Sigrist, E. de Castro, L. Cerutti, B.A. Cuche, N. Hulo, A. Bridge, L. Bougueleret, I. Xenarios. New and continuing developments at PROSITE. Nucleic Acids Res. 41 (2012) D344–D347.

[20] R.D. Finn, P. Coggill, R.Y. Eberhardt, S.R. Eddy, J. Mistry, A.L. Mitchell, S.C. Potter, M. Punta,M. Qureshi, A. Sangrador-Vegas, G.A. Salazar, J. Tate, A. Bateman. The Pfam protein families database: towards a more sustainable future. Nucleic Acids Res. 44 (2016) D279–D285.

[21] S. Babu, T.B. Nutman. Immunology of lymphatic filariasis. Parasite Immunol. 36 (2014) 338–346.

[22] S. Babu, T.B. Nutman. Immunopathogenesis of lymphatic filarial disease. Semin. Immunopathol. 34 (8) (2012) 847–861.

[23] M. Gnanasekar, K.V.N. Rao, Y.X. He, P.K. Mishra, T.B. Nutman, P. Kaliraj, K. Ramaswami. Novel phage display-based subtractive screening to identify vaccine candidates of *Brugia malayi.* Infect. Immun. 72 (8) (2004) 4707–4715.

[24] M.K. Sahoo, B.S. Sisodia, S. Dixit, S.K. Joseph, R.L. Gaur, S.K. Verma, A.K. Verma, A.K. Shasany, A.A. Dowle, P.K. Murthy. Immunization with inflammatory proteome of *Brugia malayi* adult worm induces a Th1/Th2-immune response and confers protection against the filarial infection. Vaccine 27 (32) (2009) 4263–4271.

[25] Y. Moreno, T.G. Geary. Stage-and gender-specific proteome analysis of *Brugia malayi* excretory-secretory products. PLoS Neglect. Trop. Dis. 2 (10) (2008) e326.

[26] Wongkamchai, W. Chiangjong, S. Sinchaikul, S.T. Chen, W. Choochote, V. Thongboonkerd. Identification of *Brugia malayi* immunogens by an immunoproteomics approach. J. Proteomics 74 (9) (2011) 1607–1613.

[27] C.P. Morris, S. Bennuru, L.E. Kropp, J.A. Zweben, Z. Meng, R.T. Taylor, K. Chan, T.D. Veenstra, T.B. Nutman, E. Mitre. A proteomic analysis of the body wall, digestive tract, and reproductive tract of *Brugia malayi.* PLoS Neglect. Trop. Dis. 9(9) (2015) e0004054.

[28] S. Bennuru, R.T. Semnani, Z. Meng, J.M. Ribeiro, T.D. Veenstra, T.B. Nutman. *Brugia malayi* excreted/secreted proteins at the host/parasite interface: stage-and gender-specific proteomic profiling. PLoS Neglect. Trop. Dis. 3 (4) (2009) e410.

[30] S. Bennuru, Z. Meng, J.M. Ribeiro, R.T. Semnani, E. Ghedin, K. Chan. Stage-specific proteomic expression patterns of the human filarial parasite *Brugia malayi* and its endosymbiont *Wolbachia*. Proc. Natl. Acad. Sci. USA. 108 (23) (2011) 9649–9654.

[31] D. Jiang, J. Malone, R. Townsend, G.J. Well, B.W. Li. Multiplex proteomics analysis of gender-associated proteins in *Brugia malayi*. Int. J. Parasiol. 42 (9) (2012) 841–850.

[32] B.W. Li, Z. Wang, A.G. Rush, M. Mitriva, G.J.Well. Transcription profiling reveals stage-and function-dependent expression patterns in the filarial nematode *Brugia malayi*. BMC Genomics 13 (2012) 184.

[33] J.P. Hewitson, Y.M. Harcus, R.S. Curwen, A.A. Dowle, A.K. Atmadja, P.D. Ashton, A. Wilson, R.M. Maizels. The secretomes of the filarial parasite, *Brugia malayi*: proteomic profile of adult excretory-secretory products. Mol. Biochem. Parasitol. 160 (1) (2008) 8–21.

[34] L.R. Ash, J.M. Riley. Development of *Brugia pahangi* in the jird, *Meriones unguiculatus*, with notes on infections in other rodents. J. Parasitol. 56 (5) (1970) 962–968.

[35] J.W. McCall, J.B. Malone, A. Hyong-Sun, P.E. Thompson. Mongolian jirds (*Meriones unguiculatus*) infected with *Brugia pahangi* by the intraperitoneal route: a rich source of developing larvae, adult filariae, and microfilariae. J. Parasitol. 59 (3) (1973) 436.

[36] G. Baggerman, E. Vierstraiete, A. De Loof, L. Schoofs. Gel-based versus gel-free proteomics: a review. Comb. Chem. High Throughput Screen. 8 (8) (2005) 669–677.

[37] U. Vanam, P.R. Prabhu, V. Pandey, G. Dakshinamurthy, M.V. Reddy, K. Perumal. Immune responses generated by intramuscular DNA immunization of *Brugia malayi* transglutaminase (BmTGA) in mice. Parasitology 136 (8) (2009) 887–894.

[38] R. Dhamodharan, S.L. Hoti, T. Sankari. Characterization of cofactor-independent phosphoglycerate mutase isoform-1 (Wb-iPGM) gene: a drug and diagnostic target from human lymphatic filarial parasite, *Wuchereria bancrofti*. Infect. Genet. Evol. 12 (5) (2012) 957–965.

[39] W. Hartmann, N. Singh, S. Rathaur, Y. Brenz, E. Liebau, B. Fleischer, M. Breloer. Immunization with *Brugia malayi* Hsp70 protects mice against *Litomosoides sigmodontis* challenge infection. Parasite Immunol. 36 (4) (2014) 141-149.

[40] R. Chandrashekar, K.C. Curtis, B.W. Li, G.J. Weil. Molecular characterization of a *Brugia malayi* intermediate filament protein which is an excretory-secretory product of adult worms. Mol. Biochem. Parasitol. 73 (1-2) (1995) 231–239.

[41] M.K. Sahoo, B.S. Sisodia, S. Dixit, S.K. Joseph, R.L. Gaur, S. Verma, A.K. Verma, A.K. Shasany, A.A. Dowle, P.K. Murthy. Immunization with inflammatory proteome of *Brugia malayi* adult worm induces a Th1/Th2-immune response and confers protection against the filarial infection. Vaccine 27 (32) (2009) 4263-4271

[42] M. Lei. The MCM complex: its role in DNA replication and implications for cancer therapy. Curr. Cancer Drug Targets 5 (5) (2005) 365–380.

[43] B.A. Dombroski, Q. Feng, S.L. Mathias. An *in vivo* assay for the reverse transcriptase of human retrotransposon L1 in *Saccharomyces cerevisiae*. Mol. Cell Biol. 14 (7) (1994) 4485–4492.

[44] M.T. Clegg, M.L. Durbin. Flower color variation: A model for the experimental study of evolution. Proc. Natl. Acad. Sci. U.S.A. 97 (13) (2000) 7016–7023.

[45] E.W. Ganko, V. Bhattacharjee, P. Schliekelman, J.F. McDonald. Evidence for the contribution of LTR retrotransposons to *C. elegans* gene evolution. Mol. Biol. Evol. 20 (11) (2003) 1925–1931.

[46] S.C. Copeland, V.H. Mann, M.E. Morales, H.B. Kalinna, B.J. Brindley. The Sinbad retrotransposon from the genome of the human blood fluke, *Schistosoma mansoni*, and the distribution of related Pao-like elements. BMC Evol. Biol. 5 (2005) 20.

[47] K. Hofmann, J. Tschopp. The death domain motif found in Fas (Apo-1) and TNF receptor is present in proteins involved in apoptosis and axonal guidance. FEBS Lett. 371 (13) (1995) 321–323.

[48] R.E. Campbell, R.F. Sala, I. van de Rijn, M.E. Tanner. Properties and kinetic analysis of UDP-glucose dehydrogenase from group A streptococci. Irreversible inhibition by UDP-chloroacetol. J. Biol. Chem. 272 (6) (1997) 3416–3422.

[49] H.Y. Hwang, H.R. Horvitz. The *Caenorhabditis elegans* vulval morphogenesis gene sqv-4 encodes a UDP-glucose dehydrogenase that is temporally and spatially regulated. Proc. Natl. Acad. Sci. U.S.A. 99 (22) (2002) 14224–14229.

[50] B.A. Davletov, T.C. Sudhof. A single C2 domain from synaptotagmin I is sufficient for high affinity Ca2+/phospholipid binding. J. Biol. Chem. 268 (35) (1993) 26386–26390.

[51] G.R. Howell, M. Shindo, S. Murray, T. Gridley. L.A. Wilson, J.C. Schimenti. Mutation of a ubiquitously expressed mouse transmembrane protein (Tapt1) causes specific skeletal homeotic transformations. Genetics 175 (2) (2007) 699–707.

[52] A. Dassein, J.C. Samuelson, J.E. Butterworth, M. Hogan, B.A. Sherry, M.A. Vadas, J.R. David. Immune evasion by *Schistosoma mansoni*: loss of susceptibility to antibody or complement-dependent eosonophil attack by schistosomula cultured in medium free of macromolecules. Parasitology 82 (3) 1981) 357–374.

[53] A. Cortés, J. Sotillo, C. Muñoz-Antolı, J. Molina-Durán, J. Guillermo Esteban,R. Toledo R. Antibody trapping: a novel mechanism of parasite immune evasion by the trematode *Echinostoma caproni*. PLoS Neglec. Trop. Dis. (2017). https://doi.org/10.1371/journal.pntd.0005773

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

   Onrapak Reamtong, Kitiya Rujimongkon, Nitat Sookrung, Yuwaporn Sakolvaree, Tipparat Thiangtrongjit, Atiporn Saeung, Suwich Thammapalo, Sumat Loymek, and Wanpen Chaicumpac*, Immunomes of *Brugia malayi* in microfilaremic humans and in permissive- and refractory-model hosts.

2. การนำผลงานวิจัยไปใช้ประโยชน์
   - เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)
   - เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)
   - เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)

   ☒ เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

   องค์ความรู้ใหม่ที่ได้นี้เป็นประโยชน์ทางด้านวิชาการต่อนักวิจัย บุคลากรทางการแพทย์ ที่สนใจทางด้านโรคเท้าช้าง นอกจากนี้องค์ความรู้ที่ได้จากโครงการวิจัยนี้

ยังนำไปใช้ในการเรียนการสอนนักศึกษาหลักสูตร Ph.D. (Tropical Medicine) วิชา TMMO 513 – Proteomics and metabolomics in Tropical Medicine ที่คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

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ภาคผนวก