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Diagnostic value of vascular endothelial growth factor and interleukin-17 in association with molecular diagnosis of *Wuchereria bancrofti* infection

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ABSTRACT

Objective: To explore effective diagnosis of *Wuchereria bancrofti* through DNA-based techniques followed by assessment of vascular endothelial growth factor concentration (VEGF-C) and interleukin 17 (IL-17) as indicators for lymphatic endothelial cell activation, proliferation and massive tissue reaction that may be a good indicator for ongoing lymphatic filariasis.

Methods: Blood samples were collected from 38 patients: 23 males (60.5%) and 15 females (39.5%) with filariasis and from controls (60 from a non-endemic and 22 from endemic areas). PCR was used to prove infection. A specific and sensitive ELISA was used to determine serum IL-17 and VEGF-C.

Results: A total of 28 patients (46.7%) were positive by PCR, while 10 patients (16.7%) were negative by PCR. Serum level of vascular endothelial growth factor was significantly high in acute cases [(2 147.00 ± 556.00) pg/mL] and in cases of early elephantiasis [(1 950.00 ± 638.00) pg/mL] and lowest in cases of late elephantiasis, endemic and non endemic controls [(1 238.00 ± 443.00), (807.11 ± 6.20) and (857.00 ± 91.50) pg/mL respectively]. Serum IL-17 was found to be significantly high in acute cases, early elephantiasis and late elephantiasis cases [(8 601 ± 1131), (7 867 ± 473) and (6 593 ± 378) pg/mL respectively] when compared to endemic controls [(3 194 ± 1 500 pg/mL)] and non endemic controls [(3 416 ± 1 101) pg/mL].

Conclusions: VEGF-C and its inducing factor IL-17 are expected to gain more importance in filariasis. Targeting such factors might ameliorate the pathology in chronic filariasis.

1. Introduction

The lymphatic system is crucial for fluid homeostasis, immune surveillance, and numerous pathological responses[1]. Many diseases may affect lymphatic vessels, of which lymphatic filariasis is of the utmost importance although it is globally considered a neglected tropical disease[2,3]. Worldwide, more than 1.1 billion people are estimated to be at risk of infection with lymphatic filariasis which is endemic in approximately 80 countries[4,5]. In 2002, the Egyptian Ministry of Health and Population reported 179 lymphatic filariasis endemic villages distributed in 8 governorates[6].

The major pathology in lymphatic filariasis is reactions to degenerating or dead adult worms, causing temporary or permanent disability with a major social and economic impact[6]. The majority of filaria-infected individuals exhibit strong pro-inflammatory immune responses resulting in lymphatic dilatation, hyperplasia, and lymphangiogenesis which are likely related to the parasite and its products[5,7]. Because both *Wuchereria bancrofti* (*W. bancrofti*) and *Brugia malayi* harbor the *Wolbachia* endosymbiont intracellularly, researches have accused *Wolbachia* as an inducer of vascular endothelial growth factor (VEGF) that could affect the lymphatic vessels. In addition, treatment with doxycycline, an antibiotic that targets *Wolbachia*, had been shown to reduce plasma VEGF concentration (VEGF-C)/VEGF receptor (VEGFR)-3 levels and improve pathology[8].

Th1- and Th2-type immune responses in Bancroftian filariasis had extensively been studied. A third subset of interleukin 17 (IL-17)-producing effector T helper cells, called Th17 cells, had been discovered and characterized[9], but the role of Th17-type cytokines have received less attention. VEGF had been shown to be upregulated by pro-inflammatory cytokines specially IL-17[7].

In the current study, we aim to explore effective diagnosis of lymphatic filariasis through DNA-based techniques followed by assessment of VEGF-C and IL-17, as indicators for lymphatic endothelial cell activation and proliferation and for massive tissue reaction that may be a good indicator for ongoing patent lymphatic filariasis in *W. bancrofti* infection.

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2. Materials and methods

Ethical approval was obtained from the ethical committee of Parasitology Department, Faculty of Medicine, Cairo University. Blood samples were collected from 38 individuals living in Kalyobeya and Monofeya governorates in Egypt that known to be endemic for *W. bancrofti*. Patients were 23 males (60.5%) and 15 females (39.5%) with filariasis. Their age ranged from 10 to 67 years. A control group consisting of 82 age and sex matched healthy individuals [60 from a non-endemic area (non-endemic controls) and 22 from endemic areas (endemic controls)] was also included. An informed consent was obtained from all participants according to the 1975 Helsinki Declaration. Each sample was divided into 2 tubes, one preserved on ethylenediaminetetraacetic acid for PCR (used for diagnosis of filarial infection) and the other sample was centrifuged and serum was collected and divided into 3 aliquots and stored at -20 ℃.

2.1. DNA extraction

DNA was prepared using a QIAamp® DNA blood mini kit (QIAGEN Co., GmbH, D-40724 Hilden) after digesting the samples with proteinase K in the supplied Buffer AL for 2 h at 56 ℃. DNA was prepared from the solution by using QIAmp spin columns in an Eppendorf microcentrifuge following the manufacturer's instructions.

2.2. The PCR

The most frequently used target for PCR is the *SspI* gene. Forward and reverse PCR primers designated NV-l (21 bp) and NV-2 (22 bp) were based on the consensus sequence of the *SspI* DNA repeat[9]. This allows amplification of a 188 bp DNA fragment for *Wuchereria* as follows: primer NV-l: 5'-CGT GAT GGC ATC AAA GTA GCG- 3' and primer NV-2: 5'-CCC TCA CTT ACC ATA AGA CAA C-3'. The PCR was performed in 50 μL of reaction mixture containing the following: Master Mix (Thermo-Sigma): 25 μL, 5 mL of 5× buffer, 2 μL forward primer (1:20), 2 μL reverse primer (1:20), *Taq* polymerase (0.2 μL), template DNA (5 μL) and completed with distilled water to 50 μL. DNA amplification was performed using thermocycler (Tpersonal thermocycler) and each set of reactions included a negative control (*i.e.*, reagent mixture without template DNA) and a positive control (reagent mixture with template DNA). The amplification procedure included initial denaturation at 95 ℃ for 5 min, 35 cycles, each consisting of 94 ℃ for 60 s, 50 ℃ for 60 s, and 72 ℃ for 60 s and an additional extension step at 72 ℃ for 10 min[10]. Amplified products were electrophoretically resolved on a 2% agarose gel, stained with ethidium bromide (0.5 μg/mL) (Sigma, Belgium) and the amplified PCR products were visualized under UV illumination.

2.3. Detection of VEGF-C and IL-17 in serum

Human VEGF-C was measured in serum samples using ELISA according to the manufacturer's instructions (Quantikine Human VEGF-C Immunoassay; R and D Systems). Briefly, diluted serum (1:5) or recombinant human VEGF-C standards were added to plates coated with mouse monoclonal antibody against VEGF-C and incubated for 2 h at room temperature. Plates were washed extensively 4 times and VEGF-C conjugate was added and further incubated for 2 h at room temperature. Then, a substrate was added and incubated for 30 min at room temperature protected from light. The reaction was stopped by the addition of 1 mol/L sulfuric acid and absorbance was measured at 450 nm on an ELISA reader. The lower detection limit of sensitivity of the assay was 13.3 pg/mL[11]. A specific and sensitive ELISA was used to determine concentrations of IL-17 in plasma (R & D Systems, Minneapolis, MN)[12].

2.4. Statistical analysis

Data were statistically described in terms of mean ± SD, and range, or frequencies (number of cases) and percentages when appropriate. Comparison of numerical variables between the study groups was done using student *t*-test for independent samples in comparing 2 groups and One-way ANOVA test with *post-hoc* multiple 2-group comparisons when comparing more than 2 groups. For comparing categorical data, *Chi*-square (χ^2) test was performed. Exact test was used instead when the expected frequency is less than 5. *P*-value less than 0.05 was considered statistically significant. All statistical calculations were done using computer program SPSS version 15 for Microsoft Windows.

3. Results

Of the study group, 28 cases were positive by PCR and 10 were PCR negative. PCR positive cases were clinically classified as: 5 cases with acute filariasis (fever, cough and dyspnea, painful lymphadenopathy), 23 cases with elephantiasis and/or hydrocele [(12 cases for less than 3 years (early elephantiasis) and 11 cases for 3 years or more (late elephantiasis)]. All the PCR negative cases were diagnosed to have elephantiasis (Table 1 and Figure 1). PCR is positive in early stages of the disease when the adult worms are alive and it becomes negative when the parasites are dead[13].

Table 1
Clinical classification of cases.

Clinical presentations	PCR positive (n = 28)	PCR negative (n = 10)
Acute filariasis	5	0
Elephantiasis and/or hydrocele	23	10
Early elephantiasis (< 3 years)	12	5
Late elephantiasis (≥3 years)	11	5

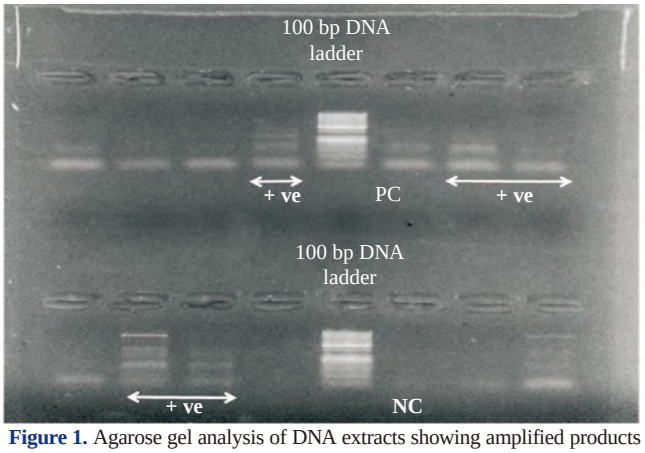

Figure 1. Agarose gel analysis of DNA extracts showing amplified products of *W. bancrofti*.
Positive samples showed amplified band at 180 bp. PC: Positive control; NC: Negative control.

Serum level of VEGF-C was significantly high (*P* value: 0.001) in acute cases [(2 147.00 ± 556.00) pg/mL] and also in cases of early elephantiasis [(1 950.00 ± 638.00) pg/mL], while it was lowest in cases of late elephantiasis, endemic and non-endemic controls [(1 238.00 ± 443.00), (807.11 ± 6.20) and (857.00 ± 91.50) pg/mL respectively] (Table 2).

Regarding serum IL-17, it was found to be significantly high in acute cases, early elephantiasis cases and late elephantiasis cases [(8 601 ± 1 131), (7 867 ± 473) and (6 593 ± 378) pg/mL respectively] when compared to endemic controls [(3 194 ± 1 500) pg/mL] and non-endemic controls [(3 416 ± 1 101) pg/mL] (*P* value: 0.000) (Table 3).

Table 2

Comparison between serum level of VEGF in different clinical presentations and control group.

Clinical presentations	Mean ± SD (pg/mL)	*P*					
		Acute	EI/H < 3 years	EI/H > 3 years	EI,-ve PCR	Endemic controls	Non-endemic controls
Acute (*n* = 5)	2 147.00 ± 556.00	-	1.000	0.001*	0.000*	0.000*	0.000*
EI/H < 3 years (*n* = 12)	1 950.00 ± 638.00	1.000	-	0.002*	0.000*	0.000*	0.000*
EI/H > 3 years (*n* = 11)	1 238.00 ± 443.00	0.001*	0.002*	-	1.000	0.100	0.896
EI,-ve PCR (*n* = 10)	963.00 ± 54.40	0.000*	0.000*	1.000	-	1.000	0.913
Endemic normals (*n* = 22)	807.11 ± 6.20	0.000*	0.000*	0.100	1.000	-	0.748
Non-endemic normals (*n* = 60)	857.00 ± 91.50	0.000*	0.000*	0.896	0.913	0.748	-

EI/H < 3 years: Elephantiasis or hydrocele for < 3 years; EI/H > 3 years: Elephantiasis or hydrocele for > 3 years; EI,-ve PCR: Elephantiasis with negative PCR.

Table 3

Comparison between serum level of IL-17 in different clinical presentations and control group.

Clinical presentations	Mean ± SD	*P*					
		Acute	EI/H < 3 years	EI/H > 3 years	EI, -ve PCR	Endemic controls	Non-endemic controls
Acute (*n* = 5)	8 601 ± 1 131	-	1.000	0.160	0.000*	0.000*	0.000*
EI/H < 3 years (*n* = 12)	7 867 ± 473	1.000	-	0.112	0.000*	0.000*	0.000*
EI/H > 3 years (*n* = 11)	6 593 ± 378	0.016	0.112	-	0.000*	0.000*	0.190
EI, -ve PCR (*n* = 10)	3 020 ± 642	0.000*	0.000*	0.000*	-	1.000	0.433
Endemic normals (*n* = 22)	3 194 ± 1 500	0.000*	0.000*	0.000*	1.000	-	0.416
Non-endemic normals (*n* = 60)	3 416 ± 1 101	0.000*	0.000*	0.190	0.433	0.416	-

EI/H < 3 years: Elephantiasis or hydrocele for < 3 years; EI/H > 3 years: Elephantiasis or hydrocele for > 3 years; EI, -ve PCR: Elephantiasis with negative PCR.

4. Discussion

Studying the role of lymphangiogenesis promoting factors is an area gaining importance in the study of lymphatic filariasis. VEGF and IL-17 are accused of being involved in or indicating filarial disease[7,14,15]. Several studies have implicated VEGF-mediated responses in the development of lymphedema and hydrocele. Some referred this to activated innate immune response following filarial infections[8,15,16].

In the present study it was observed that VEGF-C was significantly elevated in acute cases and in early elephantiasis and/or hydrocele. Level of VEGF-C was not significantly altered between previously mentioned filarial cases. However, it showed statistically significant difference from other values observed in late elephantiasis, elephantiasis with negative PCR, endemic and non-endemic control groups ($P < 0.05$). This was in accordance to Bennuru and Nutman[17], who strongly indicated an active involvement of VEGFs in lymphatic filariasis. The authors studied the level of several types of VEGF (VEGF-A and VEGF-C/soluble VEGFR-3] and had observed their association with filarial disease. It had been reported that filarial antigens and plasma from filaria-infected individuals have the capacity to induce human lymphatic endothelial cells to undergo proliferation and differentiation, a process mediated by either excreted or secreted parasite proteins and lymphangiogenesis promoting factors as VEGF. On the contrary, plasma from non-infected individuals or chronic cases induced nothing[17]. By estimating serum level of the recently studied cytokine, IL-17, it was noticed that it was significantly higher in acute cases and in early and late elephantiasis and/or hydrocele than in other individuals (elephantiasis with negative PCR, endemic and non-endemic control groups). Accordance in the elevated levels of the estimated serum factors (VEGF and IL-17) in the present study within different patient groups was interestingly noticed.

Babu *et al*. indicated the importance of proinflammatory cytokines especially IL-10 and Il-17 in regulation of VEGF[7]. They showed that filarial antigen-mediated proinflammatory cytokine induction was a characteristic feature of chronic filarial lymphedema and hydrocele. They added that these cytokines and subsequently VEGF were significantly lower in subclinical (or asymptomatic) infection. It had also been shown that IL-17 could mediate lymphatic damage by inducing production of various angiogenic and lymphangiogenic factors leading to perturbations in lymphatic endothelial system function ending in lymphatic dilatation and lymphedema. Moreover, presence of elevated levels of lymphangiogenic factors had been shown to be associated with the severity of lymphatic pathology[18,19].

Honorati *et al*.[20] and Lohela *et al*.[21], explained the importance of IL-17 and the subsequent stimulation of VEGF formation as important regulatory growth factors for vascular and lymphatic endothelial systems that can also promote both angiogenesis and lymphangiogenesis by interacting with cognate receptors (VEGFR-1, -2, and -3) on endothelial cells. This was also supported by Wu and Liu[22] and Debrah *et al*.[16] who showed that generation of new lymphatic vessels from the pre-existing lymphatic system is strongly affected by VEGF that has a crucial role as an angiogenic/lymphangiogenic factor in microfilaraemic patients who developed elephantiasis. On the contrary, those who didn't develop elephantiasis had a significant lower level of VEGF.

In our study, patients with elephantiasis and negative PCR had significantly lower VEGF-C. Babu *et al*. arose an important

point when reporting that filarial antigens induce differential production of VEGF-C, illustrate certain similarities and (perhaps) differences in lymphatic response to different filarial antigens[7]. This may explain the old debate why some infected individuals develop elephantiasis while other infected individuals with proved microfilaraemia escape lymphatic affection.

Another study proved that antifilarial treatment with doxycycline leads to reduction of plasma levels of VEGF being associated with amelioration of dilated lymphatic vessels and with an improvement of pathology in lymphatic filariasis patients[18,23].

Researchers demonstrated that VEGFs being an important angiogenic/lymphangiogenic factor are elevated in vascular diseases, tumors, lymphedema and lymphangioleiomyomatosis; however, elevated VEGF-C is particularly associated with changes in the lymphatics in filarial infections. This may be due to the fact that filarial antigens are strong inducers for VEGF-C[7,16].

In conclusion, the studying level of VEGF-C and its inducing factors as IL-17 is expected to gain more importance in the fields of lymphatic diseases especially filariasis. Probably targeting such factors and their pathway might potentially ameliorate the pathology in chronic filariasis.

Conflict of interest statement

We declare that we have no conflict of interest.

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