Assessment of the Role of Serum and Urine Eosinophil Cationic Protein in Diagnosis of *Wuchereria bancrofti* Infection

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Abstract: Eosinophils participate in the complex regulatory system that mediates inflammatory responses and parasitic infections. when activated eosinophils degranulate, they release Four highly basic proteins which include Eosinophil cationic protein (ECP) as a major constitutent of secondary eosinophilic granules . In this study, the ECP levels were measured in both serum and urine of patients with lymphatic filariasis (LF) with different presentations and compared to their levels in normal individuals living in endemic areas (endemic normals), asthmatic patients as an example of allergic condition and normal individuals living in non-endemic areas (non-endemic controls). Serum ECP was found to be significantly elevated in all filarial cases with marked elevation in acute cases reflecting the ongoing pathological process. The asthmatic patients and to a lesser extent the endemic normals showed an elevated mean serum ECP. In addition, urinary levels of ECP were significantly elevated in the chyluria cases reflecting the pathological condition affecting the urinary tract. In conclusion: Measurement of serum and urine ECP can help in the diagnosis of infection with Wuchereria bancrofti and can also be used in the follow-up of pathological changes in lymphatic filariasis as its levels reflect the hidden tissue eosinophilic inflammatory reaction associated with this disease. [Journal of American Science 2010; 6(9):515-523]. (ISSN: 1545-1003).

Key Words: *Wuchereria bancrofti* , Lymphatic filariasis, Eosinophil cationic protein, Serum, Urine.

1. Introduction

Lymphatic filariasis (LF) affects around 120 million people worldwide and Wuchereria bancrofti (W. bancrofti) is responsible for 90% of LF cases. It represents a broad clinical spectrum, including acute and chronic filariasis (mainly elephantiasis and hydrocele) [(1)]. however, acute cases may remain asymptomatic until adult worms settle in the lymphatic tissue. Also a large number of microfilaraemic cases may be asymptomatic and are called endemic normals. So in most endemic communities the number of people who have elephantiasis is relatively small compared with those who suffer from acute disease even if asymptomatic[(2)].

Also acute filariasis can occur without peripheral microfilaraemia and may be misdiagnosed as malaria or other tropical diseases, which could lead to incorrect treatment and a waste of health care resources, which puts us in need of other tests or parameters that aid in the diagnosis and follow up of such cases[(3)].

The granules of the human eosinophilic granulocytes contain several highly cationic proteins which upon activation and stimulation of the cells are secreted. One of these proteins is the eosinophil cationic protein (ECP). ECP is a highly basic and potent cytotoxic single-chain zinc-containing protein. ECP appears to be involved in defense against parasites and in the tissue damage seen in subjects with allergic and inflammatory diseases [(4)].

ECP has been proven to be useful in monitoring many diseases. Interestingly, recent studies have evaluated this protein as an indicator of the activity or the progress of the diseases as done by some cardiologists who assessed the association between baseline serum levels of ECP, a sensitive marker of eosinophil activation, and recurrence of clinical events after stent implantation[(5)].

This toxic cationic protein released from the eosinophils in filariasis patients has been found to be able to damage the larval forms of *W.bancrofti* and is also correlated with the degree of eosinophilia. The level of the granule proteins ECP in blood appears to reflect the ongoing parasitic disease process [(6)].

The measurement of urinary levels of ECP similarly contributes to the diagnosis and prognosis of the disease in chyluria patients [(7)]. Serum and urine levels of ECP have been measured in patients with filariasis. ECP appears to be a marker of the eosinophils in blood and tissue[(8)].

The main objective of this study was to elucidate the characteristics of ECP as a marker of morbidity in *W. bancrofti* infection, by comparing ECP levels with other allergic and inflammatory diseases such as bronchial asthma.

2. Patients and Methods:

This study, which was conducted between October 2007 and May 2009, included 50 serum and urine samples collected from men, women and children of both sexes with history or clinical picture

suggestive of filariasis, aged between 8 and 74 years old.

The above patient group, was compared to 15 endemic controls, i.e. lifelong residents, from the same geographic area (endemic normals) and also compared to 15 filariasis negative individuals from a non-endemic area (non-endemic controls). Samples from 9 asthmatic patients were also collected to compare between the ECP levels of parasitic and non-parasitic allergic patients (after exclusion of parasitic infections in these groups).

Field teams consisting of two physicians and a local health worker collected samples after obtaining informed consent from people living in the villages of Marsafa and Dolhomo in Kalyobeya and Monofeya governorates respectively, both of which are areas endemic for *W. Bancrofti*.

The definitive parasitologic diagnosis for lymphatic filariasis is the demonstration of microflariae in blood samples. However, this gold standard test is restricted by the requirement for nocturnal blood collection and lack of adequate sensitivity. Detection of circulating antigens on the other hand is commercially available. If used as a single test, its usefulness is limited for *W. bancrofti*, so it must be accompanied by another serological test to increase its sensetivity. In addition, microfilaremia and antigenemia may develop from months to years after exposure[9].

Blood and urine sampling:

The blood was collected by standard venipuncture and divided between a plain vacutainer tube and one containing EDTA.

All samples were tested for the presence of IgM and IgG antibodies raised against *W. bancrofti* using the OnSite Filariasis IgG/IgM Rapid Test-Cassette (Serum / Plasma) (CTK Biotech Inc., CA. USA). This is a lateral flow chromatographic immunoassay for the simultaneous detection and differentiation of IgG and IgM anti-lymphatic filarial parasites (*W. Bancrofti and B. Malayi*) in human serum or plasma. The test cassette utilizes recombinant *W. bancrofti and B. malayi* common antigens conjugated with colloid gold (Filariasis conjugates) and rabbit IgG-gold conjugates. Any reactive specimen with the OnSite Filariasis IgG/IgM Rapid test must be confirmed with alternative testing method(s) as some specimens containing unusually high titer of heterophile antibodies or rheumatoid factor may affect expected results so the results obtained with this test should only be interpreted in conjunction with other diagnostic procedures and/or clinical findings. Antibody detection provides an early means to detect filarial parasitic infection. The presence of anti-filarial IgM antibodies suggests current infection, whereas, anti-filarial IgG corresponds to late stage of infection or past infection[9].

For diagnosis of active infection in bancroftian filariasis or presence of hidden microfilaraemia in endemic normals, serum samples were examined for *W. bancrofti* antigen using the NOW® Filariasis test, a rapid in vitro immunochromatographic test (ICT) (Binax Inc., Portland, U.S.A) for the qualitative detection of *W. bancrofti* antigen in whole blood, serum or plasma. The test utilizes a polyclonal antibody (PAb) and a monoclonal antibody (MAb) specific for *W. bancrofti*. This test is an important and continuously evolving challenge to filariasis-endemic countries and to health personnel[10].

A sterile lancet was used to collect fresh blood to fill a capillary tube for the ICT test for detection of filarial antigen. ICT was performed as per the manufacturer's instructions (NOW, ICT filariasis kits, Binax, Portland, ME, USA). The patient's left index finger was cleaned with ethanol, and then punctured by using a sterile lancet. The initial sample of blood was removed using a cotton swab, and sufficient fresh blood was then collected to fill a 100- ql capillary tube. The blood was then transferred from the capillary tube to the pad on the ICT card and then sealed. The results of each ICT were read after 15 min [11].

After performing the OnSite Filariasis IgG/IgM test and the ICT test for detection of filarial antigen for both suspected patients and endemic normals, the collected samples were carried on ice until brought to the laboratory where CBCs were performed on the EDTA blood samples and serum was obtained from the samples containing no additive by centrifugation at 1000×g for 10 min. Samples of spontaneously released urine were collected in plastic containers and centrifuged to remove particulates. Aliquots of serum and urine were then frozen at – 20° C until the time of assay.

Upon confirmation of active infection or LF and clinical classification of 39 cases[12], all patients were subsequently treated with diethyl carbamazine (DEC).

Determination of eosinophilic cationic protein in plasma and urine:

ECP levels were measured in serum and urine samples of all studied groups by a human ECP ELISA kit (USCN LIFE Science and Technology CO., LTD, China).

Briefly, samples were added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for ECP. Next, Avidin conjugated to Horseradish

Peroxidase was added to each microplate well and incubated. A substrate solution was added to each well. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm±2 nm. The concentration of ECP in the samples was then determined by comparing the O.D. of the samples to the standard curve.

Statistical analysis:

Data was coded and entered using the statistical package SPSS version 15. Data was summarized using number and percent for qualitative variables and mean and standard deviation for quantitative variables.

Comparison between groups was done using the Chi square test for qualitative variables and analysis of variants (ANOVA) with multiple comparison post hoc test for normally distributed quantitative variables, while the non-parametrical Kruskal-Wallis test and the Mann-Whitney test were used for normally distributed quantitative variables.
Correlations were done to test for linear relation between quantitative variables. P-value less than or equal to 0.05 were considered statistically significant.

3. Results

In this work, which included 50 participants suggestive of having filariasis, 39 gave positive results by the *OnSite* Filariasis IgG/IgM Rapid Test-Cassette (31 were positive for filariasis IgG only, 6 were positive for filariasis IgM only and 2 were positive for both IgM and IgG).

The 39 positive cases were classified clinically as follows: (6 cases of acute filarialiasis, 20 cases of chronic elephantiasis, 7 cases of hydrocoele and 6 cases of chyluria).This is summarized in table (1).

Table (1): Positive cases for IgG, IgM or circulating filarial antigen (CFA).

No of suggested cases (50)	Negative (11)	Positive (39)					
		Positive IgG only: (31 cases)			**Positive IgM only:** (6 cases)		**Positive IgM & IgG** (2 cases)
		20 cases of elephantiasis	7 cases of hydrocele	4 case of chyluria	5 acute cases	1 case of chyluria	1case of chyluria, 1 acute case
CFA		Negative	Negative	Negative	Positive: (5 cases)	Negative	Positive in acute, Negative in chyluria

Regarding the endemic normals, only 3 of the 15 showed positive IgG and all were negative for IgM and CFA.
Collectively, the study group was formed of 78 samples. Serological determination of the granule protein ECP was done for:
- 39 patients (22 females and 17 males), all infected with bancroftian filariasis and these included: (6 cases of acute filariasis, 6 cases of chyluria, 7 cases of hydrocele and 20 cases of elephantiasis).

These were compared with ECP levels in:
- 9 asthmatic patients.
- 15 controls from non-endemic areas (non-endemic controls).
- 15 controls from endemic areas (endemic normals).

In the representative sample, the total leucocytic count and peripheral blood eosinophils were counted. Demographic and laboratory data of the studied groups can be presented in (Table 2).

Table (2): Demographic and laboratory data of the studied groups:

Parameter	*Group*	*N*	*Mean ± Standard deviation*
Age (ys)	Acute	6	15 ± 6.5
	Chyluria	6	23 ± 8.4
	Elephantiasis	20	52 ± 13.3
	Hydrocele	7	44 ± 10.2
Duration (ys)	Acute	6	1.25 ± 1.044
	Chyluria	6	3.50 ± 2.887
	Elephantiasis	20	21.04 ± 10.635
	Hydrocele	7	5.33 ± 2.774
Absolute eosinophil count	Control (non-endemic)	15	205.5 ± 55.6
	Control (endemic normal)	15	255.5 ± 46.6
	Asthma	9	417.3 ± 65.3
	Acute	6	804.3 ± 75.5
	Chyluria	6	360.8 ± 34.7
	Elephantiasis	20	208.4 ± 49.5
	Hydrocele	7	443.7 ± 57.9
ECP in serum ng/ml	Control (non-endemic)	15	28.30 ± 1.58
	Control (endemic normal)	15	35.57 ± 2.86
	Asthma	9	39.57 ± 2.94
	Acute	6	89.06 ± 7.52
	Chyluria	4	54.85 ± 5.12
	Elephantiasis	20	43.98 ± 4.94
	Hydrocele	3	47.93 ± 3.05
ECP in urine ng/ml	Control (non-endemic)	15	1.45 ± 0.19
	Control (endemic normal)	15	1.43 ± 0.18
	Asthma	9	1.51 ± 0.23
	Acute	6	1.38 ± 0.09
	Chyluria	4	11.12 ± 1.07
	Elephantiasis	20	1.44 ± 0.16
	Hydrocele	3	1.50 ± 0.10

In the previous table, it can be noticed that the mean serum ECP levels were slightly elevated in the 15 endemic controls compared to the non-endemic controls (non significant).

The asthmatic patients showed significantly elevated mean serum ECP levels in comparison to non-endemic controls ($p = 0.05$). Also in the hydrocele, elephantiasis and chyluria patients, there was a higher extent of elevation of serum ECP (about 1.5 to 2 folds higher than non-endemics). As for the acute cases, the ECP level in their serum was highly elevated (up to 3 folds higher than non-endemic controls). However, the only cases showing elevated urine ECP levels were the chyluria patients who had markedly elevated mean ECP levels in their urine (about 7 folds higher than control levels).

Comparing the serum ECP levels in the different groups, as shown in table (3) and figure (1), revealed that the mean circulating concentration of serum ECP in acute cases was elevated (89.07±7.5203) with symptomatic significance ($P<0.01$) when compared to different groups.

Chyluria patients showed a significant elevation of serum ECP as compared to control groups and compared to the bronchial asthma group.

Elephantiasis patients also showed a significant elevation of serum ECP when compared to non-endemic controls, but no significant difference was found when compared to endemic normals.

Lastly, the hydrocele cases showed a significant elevation in ECP levels (47.93 ± 3.05) when compared to non-endemic controls (P=0.01) and a borderline statistical significance when compared to endemic normals (P = 0.05).

On comparing urine ECP levels in different groups, which can be shown in table (4) and figure (2), the following was observed: the mean circulating concentration of ECP in the urine was significantly elevated (11.125 ± 1.0720) in chyluria cases when compared to all other groups including control groups and cases.

Table (3): Serum level of ECP in all studied groups:

Group	*Control (non-endemic)*		*Control (endemic normals)*		*Asthma*		*Acute*		*Chyluria*		*Elephantiasis*		*Hydrocele*	
	ECP (ng/ml)	*P*	*ECP (ng/ml)*	*P*	*ECP (ng/ml)*	*P*	*ECP (ng/ml)*	*P*	*ECP (ng/ml)*	*P*	*ECP (ng/ml)*	*P*	*ECP (ng/ml)*	*P*
Control (non-endemic)				0.70		0.05*		<0.01*		<0.01*		0.050*		0.01*
Control (endemic normals)		0.70				1.0		<0.01*		0.01*		0.071		0.05*
Asthma		0.05*		1.00				<0.01*		0.03*		1.00		0.70
Acute	28.30 ±1.58	<0.01*	35.57 ±2.86	<0.01*	39.57 ±2.94	<0.01*	89.07 ±7.52		54.85 ±5.12	<0.01*	43.98 ±4.94	<0.01*	47.93 ±3.05	<0.01*
Chyluria		<0.01*		0.01*		0.03*		<0.01*				0.050*		0.70
Elephantiasis		0.05*		0.07		1.0		<0.01*		0.05*				1.0
Hydrocele		0.01*		0.05*		0.70		<0.01*		0.70		1.00		

*means significant P-value.

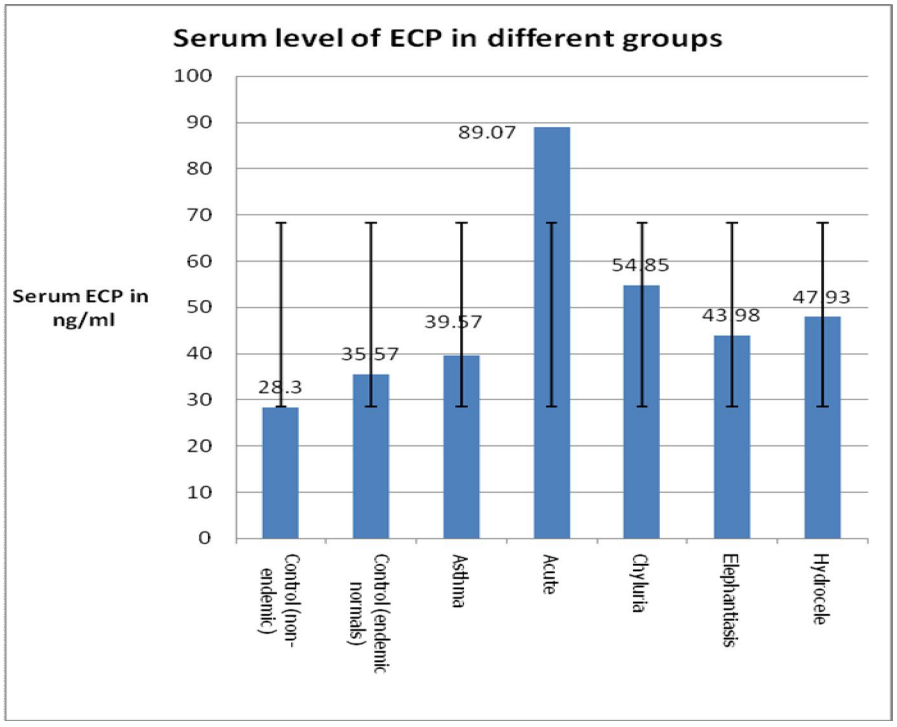

Fig. (1): Serum level of ECP in different groups.

Table (4): Urine level of ECP in all studied groups:

Group	Control (non-endemic)		Control (endemic normals)		Asthma		Acute		Chyluria		Elephantiasis		Hydrocele	
	ECP (ng/ml)	P	ECP (ng/ml)	P	ECP (ng/ml)	P	ECP (ng/ml)	P	ECP (ng/ml)	P	ECP (ng/ml)	P	ECP (ng/ml)	P
Control (non-endemic)	1.45 ±0.19		1.43±0.18	1.00	1.51 ±0.23	1.00	1.38 ±0.09	1.00	11.12 ±1.07	<0.01*	1.44 ±0.16	1.00	1.50 ±0.10	1.00
Control (endemic		1.00				1.00		1.00		<0.01*		1.00		1.00
Asthma		1.00		1.00				1.00		<0.01*		1.00		1.00
Acute		1.00		1.00		1.00				<0.01*		1.00		1.00
Chyluria		<0.01*		<0.01*		<0.01*		<0.01*				<0.01*		<0.01*
Elephantiasis		1.00		1.00		1.00		1.00		<0.01*				1.00
Hydrocele		1.00		1.00		1.00		1.00		<0.01*		1.00		

*means significant P-value.

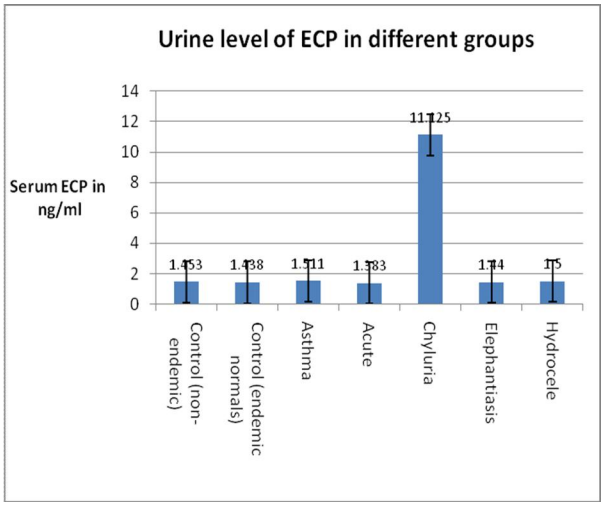

Fig. (2): Urine level of ECP in Different groups. A significant positive correlation was found between the mean serum ECP and the eosinophil count (P-value <0.01, r=0.60), this can be seen in figure (3).

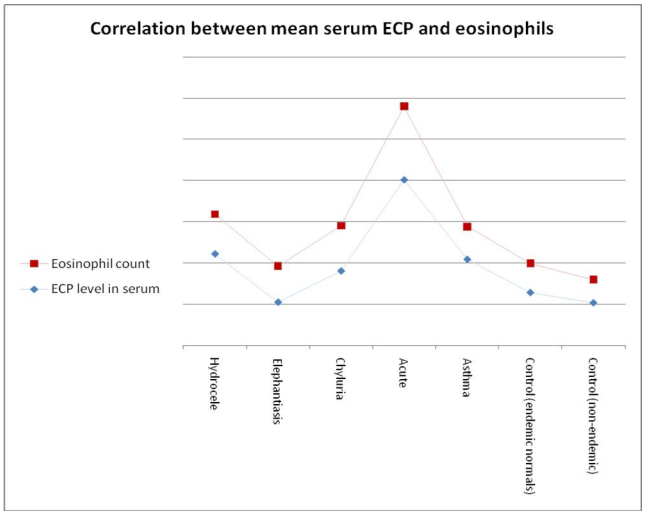

Fig.(3): Correlation between mean serum ECP and eosinophil count

4. Discussion

The aim of this study was to examine whether serum as well as urine levels of ECP are a useful clinical and chemical parameter in bancroftian filariasis compared to other allergic diseases such as asthma and compared to individuals living in endemic areas (endemic normals). Studying the ECP level in these groups showed that levels of ECP were generally higher in filariasis infected individuals (especially in acute cases) than in those with bronchial asthma or in endemic normals.

In the present study, the acute filariasis cases were associated with eosinophilia, while only some of the chronic filariasis cases had mild eosinophilia. This shows that an association with an elevated peripheral eosinophil count in *W. bancrofti* infection was found mainly in non-symptomatizing acute cases (absolute eosinophil count= 804.3).

This is supported by Gopinath, et al. [13] who opined that it may be helpful, sometimes, to rely on the eosinophil count as a marker for the severity of parasitic infection, but that it is frequently misleading. If the eosinophil count is to be used as a marker, the total eosinophil count must be determined rather than the percentage as this may vary greatly according to the total leucocytic count. Patients must also be immunocomptent to be able to rely on the eosinophil count and another important thing is that downregulation of eosinophil levels usually occurs in longstanding chronic filarial cases. Thus patients with chronic filariasis may have normal or minimally elevated peripheral eosinophil count.

The present results also are in agreement with the findings of Noguchi, et al. [14] who recorded a statistical significance in elevated serum ECP levels in flilarial patients compared to normal individuals. They found that in acute filariasis serum ECP levels may increase up to (96.8 ng/ml). This may reflect an ongoing pathological process causing tissue eosinophlia, or acute filarial infection on top of chronic filariasis.

Tischendorf, et al. [15] studied several eosinophilic proteins and found that, eosinophilic granular proteins are helminthotoxic in vitro and are elevated in filarial infections. They added that filaricidal activities, however, are not equivalent among the effector proteins; ECP has greater in vitro microfilarial toxicity than other eosinophilic granular proteins, thus their results are in agreement with this work.

To our knowledge, only a few studies have been performed on ECP levels in *W.bancrofti* infection, but this study is also supported by the work done by Tischendorf et al. [7] on onchocerciasis. They stated that the serum level of ECP shows a significant correlation with activated eosinophils which are the only leucocytes containing this protein. They also showed that the highest ECP levels in serum and urine were observed in the hyperactive sowda form which is an acute form of onchocerciasis.

In the present study, the elevated ECP level is not always associated with peripheral eosinophilia as tissue eosinophilia itself is supposed to be the cause of elevation of ECP level in serum with absence of peripheral eosinophilia. This can be shown in chronic cases as in elephantiasis where absolute eosinophil count was not elevated (208.4), while serum ECP was elevated (43.980 ng/ml) with ($p= 0.053$) compared to control non endemic group.

This is supported by Wildenburg et al. [16] who said that the lack of positive correlation of ECP with the peripheral eosinophil counts in some cases may indicate that serum granule proteins may also be derived from eosinophils in tissues like lymph nodes or bone marrow. The circulating pool of eosinophils represents only a minor part of the eosinophil mass. Most of the clearance of helminthes apparently occurs in the liver, spleen, lungs, gut, skin and lymph nodes in association with mixed cell inflammatory reaction with a large number of macrophages and eosinophils surrounding the dying microfilarae.

In this work, ECP levels in urine showed a considerable elevation in the chyluria patients as compared to other groups (ECP $=11.12 \pm 1.07$) ($p <0.01$). This was referred to the degree of pathological changes in the affected lymphatics of the urinary system. Pathological changes, particularly at an early stage of infection with *W.bancrofti*, can therefore be suggested by measuring the ECP level in urine which may be useful for early management of such cases.

This was in accordance with Sharma and Hemal [17] who studied urinary ECP level in chyluria patients, in relation to its level in other filarial patients. They stated that significant rise of ECP level in urine was obvious even before imaging detectable urinary tract pathology, denoting that ECP may therefore provide important information on the evolution of *filaria*-associated urinary tract morbidity.

Thus this study shows that serum ECP in patients with filariasis and urine ECP in chyluria patients, is a tool that may help or support the diagnosis of filariasis, also it can be used for indicating and assessing the pathogenic progression related to tissue eosinophilia in such cases even in absence of peripheral eosinophilia.

In this study it was found that serum levels of ECP were elevated in the bronchial asthma patients and in the endemic normals. In both groups a significant correlation was observed between serum levels of ECP and blood eosinophil counts, however

values of ECP in the two groups did not exceed mean of 40 ng/ml, which was significantly lower than levels observed in patients with filariasis (P<0.01).

This is also in accordance with the results of Sugai et al., [18] who showed that ECP levels were found to be elevated in the serum of patients with bronchial asthma and atopic dermatitis. However he observed a significant correlation in both diseases between serum levels of ECP and blood eosinophil counts, which may have been due to the selection of a group of patients who have an ongoing active disease that caused peripheral eosinophilia.

This was supported by Koh et al. [19] who studied ECP as one of the members of the eosinophil-associated ribonuclease family which can regulate fibroblast activity, modulate airway mucus secretion, and interact with the coagulation and complement system. They measured ECP in serum, plasma, sputum, saliva and broncho-alveolar lavage fluids but serum and sputum are the most established. They concluded that despite its limitations, ECP remains potentially useful in asthma management especially when combining it with other markers of asthma.

In 2006, Moneret-Vautrin[20] considered the value of measuring ECP levels for the diagnosis of various diseases where an eosinophil mediated tissue inflammation plays a role. He found that in parasitic diseases the pathogenic progression may be accurately assessed, especially if other serological tests are less indicative. In the event of isolated hypereosinophilia, the ECP assay may clarify if there is an underlying parasitological pathogen causing the disease as ECP is higher in helminthic infections.

Other researchers found that serum ECP was significantly increased in elephantiasis patients. It has also been evaluated in another helminth disease, namely schistosomiasis, and was found to be of potential interest in terms of follow-up of morbidity in this parasitic disease [21].

Thus this study showed that serum and urine concentrations of ECP appear to mirror the functional activity of eosinophils, so there is marked increase of this protein in the serum and less increase in the urine which seems to reflect the activation and augmented turnover rate of the eosinophils even in the absence of peripheral eosinophilia.

5. Conclusion:

ECP has been proven to be useful in monitoring and assessing the severity of filarial infections and indicating the severity of certain inflammatory conditions. The measurement of serum ECP presents an advantage over subjective clinical measures, which are prone to inconsistencies due to the broad variability of individual investigator and patient assessments

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7/21/2010