Poly Acrylamide Gel Electrophoresis of Serum Protein, Application to Kala-azar Patients

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Abstract

Sera samples were collected from 60 children aged 4-60 months, all were clinically and serologically proven cases of visceral leishmaniasis, as well as from 10 healthy children, all were seronegative with no history of parasitic infection who serve as a control during the study. Serum total protein and albumin were measured and compared between the control and visceral leishmaniasis patients. Serum protein profiles have been investigated using the conventional sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE). Serum of control group showed the specific protein pattern with five protein bands, while serum protein profile in visceral leishmaniasis patients revealed three groups of electrophoretic banding patterns. , 50% showed twelve bands, 36.66% of the patients showed nine bands and 13.33% showed ten bands. At least four of these bands were found to be common among the infected groups which may be of diagnostic value and required further investigations. The three different electrophoretic patterns groups may be correlated with the previous epidemiological observations in respect of different clinical presentation of the disease and different response the chemotherapeutic agents at many endemic areas around the world.

Key words: Protein band, protein patterns, serum protein, sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE), visceral leishmaniasis.

الخلاصة

جُمِعتْ عينات المصل مِنْ ٦٠ طفلٍ عراقي بعمرِ ٤-٦٠ شهر، كُلّ الحالات اثبتت سريرياً ومناعيا الاصابه بداء اللشمانيات الاحشائيه ، ومِنْ ١٠ أطفالِ اصحاء، كَانتْ نتائجهم سالبه مناعيا وبدون تأريخ عدوى مرض طفيلي اخر، استخدمت هذه المجموعه كسيطرة أثناء الدراسةِ.تم قياس البروتين الكلي والزلال في مصل المصابين والاصحاء وتمت المقارنه بين المجموعتين. تم التحري عن بروتينات مصلِ الدم بإستعمال تقنية الترحيل الكهربائي التقليدي (هلام متعدد الاكريل امايد- SDS. اظهر مصلُ المجموعة القياسيةِ نمطَ البروتينِ الاعتيادي بخمسة حزم ، بينما اظهر مصلِ مرضى اللشمانيا الاحشائيه وجود ثلاثة مجموعاتِ مختلفة الأنماط وقد كانت كما يلي: ٥٠ % اظهر إثنتا عشْرة حزمه و٣٦.٦٦ % اظهر تسعة حزم و١٣.٣٣ % اظهر عشْرة حزم. كما كشفت هذه الدراسه على الأقل وجود اربعه مِنْ هذه الحزم شائعه بين المجموعاتِ المُصَابةِ. و قَدْ تُكُون لهذه الحزم الاهميه في تشخيص المرض مما يستدعي اجراء دراسات اوسع واعمق والتحقيقاتِ الأخرى المطلوبةِ للكشف عن نوعيه هذه البروتينات المشتركه ضمن المجاميع المختلفه. هذه المجاميع الثلاثه من الانماط البروتينيه قَدْ تُرْبَطُ بالدراسات الوبائيه السابقة وخاصة في المناطق التي يكون المرض فيها متوطنا فيما يتعلق بالعوارض السريريه المختلفِه مِنْ المرضِ والاستجابه المختلفِه للعلاج في هذه المناطق.

Introduction

Leishmaniasis forms a whole group of parasitic tropical diseases which spread by the bites of many different species of sand fly producing at least 5 distinct diseases with different symptoms [(1)]. Visceral leishmaniasis (VL) or (Kala-azar) is one of the most serious forms, it is life threatening, causing very high mortality rates unless treated [(2)], and even with the treatment mortality can be from 5-15% and even higher if treatment is delayed [(3)].VL is a systemic disease of the mononuclear pathogenic system. The reticuoendothelial hyperplasia that follows infection with *Leishmania donovani* affects the spleen, liver, mucosa of the small intestine, bone marrow, lymph nodes and other lymphoid tissues [(4,5)]. Hypoalbuminaemia is associated with oedema and other features of malnutrition [(6)]. Hyperglobulinaemia mainly polyclonal immunoglobulin G (IgG) is common but does not seem to have a clear pathological role in VL [(7)].Complement activation may contribute to anaemia and immune complexes are formed, but nephritis is rare [(8)]. Secretory IgM as well as IgG (specific and non-specific) were found to contribute to disease susceptibility and appear to act in part through the activation of complement a+nd generation of C5a [(9)].

These findings extend earlier murine VL studies [(10)] and indicate that a polyclonal B cell response is an early and intrinsic feature of VL, which helps to establish and maintain infection in the mammalian host [(11)]. The presence of high titers of circulating immunocomplexes has been reported in patients with visceral leishmaniasis [(10,12)].

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Signs of activation of the complement cascade were observed in a study of patients with visceral leishmaniasis and, in 43.4% of the cases, it was associated with a simultaneous reduction in the levels of C3 and C4, along with changes in CH100, suggesting activation of the classical pathway [13]. On the other hand, a reduction in C3 levels in patients with visceral leishmaniasis was observed in other study, suggesting activation of the alternative pathway [14,15] Corroborating those data, Stebut [16] observed activation of the complement system in experimental models of leishmaniasis, especially by opsonization of the surface of the parasite with C3b. Studies on serum protein in VL patients indicated that the total serum proteins dropped below normal limits in 80% of the cases [8]. The main decrease being observed in albumin fraction which dropped to about half of the total control values, a moderate increase in γ-globulin fraction and an increase in the relative concentration of α-globulin were also noted [7]. The aim of this study is to investigate the changes in serum protein fraction of patients with VL.

Patients and Method

This study was divided into two groups:

1. Patients: including 60 children aged 4-60 months, admitted to paediatric ward of Al-Kadhimiyah Teaching Hospital-Al-Nahrain University/ Baghdad, from December 2006 - Febriwary 2009. All were proven cases of Kala-azar by: clinical manifestation of fever, hepato-spleenomegally, and the demonstration of the parasite - the amastigotes – from direct smear of bone marrow and serological diagnosis by indirect immunofluorescence antibody test IFAT. The diagnosis was under the supervision of Dr. Shatha Hussain (paediatrician) and Dr. Rabab Al-Sa'adi (dermatologist).
2. Control: consist from 10 healthy children all were seronegative.

Blood samples

Two milliliters of venous blood were collected from patients and control in plain plastic tubes and the serum was obtained by centrifugation at 4°C, 400 × G for 10 minutes and used freshly for electrophoresis. Total serum protein, albumin was measured for each sample according to Biurate [17], Wooten [18] respectively. The protein electrophoresis was dependent on the conventional polyacrylamid gel electrophoresis PAGE as described by Fehrnstrom and Morberg [19].

Electrophoresis of serum protein

The process of electrophoresis was carried out as described by Fehrnstrom and Morberg [19].Serum samples were transported on a horizontal slab of poly acrylamide gel of (7.5% w/v) .

Electrophoresis:

The multiphor cooling plate was thermo stated to not more than 10ºC. Pre-electrophoresis was performed at 50 mA for 30 minutes. Then, samples were applied as (9µl sample +1µl bromophenol blue), then concentrated for 5-10 minutes with a current of 20 mA. The voltage was adjusted between 350-400 volts and the current was around 50 mA. The electrophoresis time of each run was about 4 hours. After running of the electrophoresis process, Coomaise brilliant blue (0.25% w/v) was used for staining of the bands. Visualization of the bands was carried out after destaining by (ethanol+acetic acid+distilled water) at (3:1:6) respectively. Migration distance of the calibration proteins were measured after staining the protein bands, also relative mobility (RM) was measured according to the following equation:

$$RM = \frac{Distance\ of\ protein\ migration}{Distance\ of\ dye\ migration}$$

The calibration curve was constructed by plotting relative mobilities (RMs) versus logarithmic molecular weights for calibration proteins figure 1. The molecular weight of proteins of interest was determined from the position of its RM value on the calibration curve.

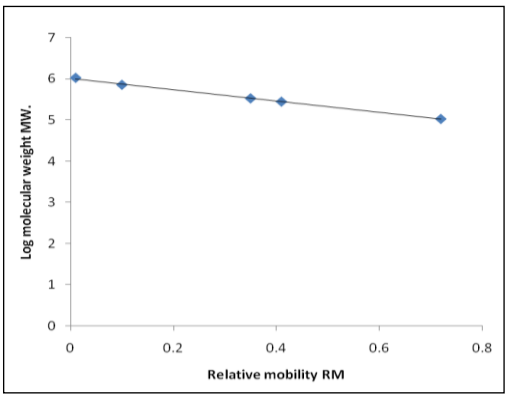

Figure 1 : Standard curve for approximate estimation of molecular weights of various proteins in serum samples.

Results

Total protein

Serum total proteins were found to be elevated in 30 patients (50% of the samples) compared with the control, while the remaining 30 (50%) were found to be within normal range . On the other hand, Serum albumin was found to be markedly reduced in 58 patients (96.66% of the samples) compared with the control, except two patients (3.33%) were found to be within normal range table 1.

Table 1: Serum total protein and albumin of normal and patients with Kala-azar.

Groups		Number of samples	Total protein g/dl±SD	% of high levels total protein	Albumin g/dl±SD	% of high levels albumin
Control		10	5.3±1.2	----	5.2±0.12	---
patients		60	10.2±0.5	50	3.5±0.62	58

Protein electrophoresis (serum protein profile)

Conventional polyacrylamide gel electrophoresis has been used to differentiate between protein patterns in serum of normal and patients with Kala-azar [(20)]. Serum of control group has a specific protein pattern with five protein bands (albumin, α_1, α_2, β_1, γ), the relative mobility values (RM) have been ranged from (0.015-0.72) table 2 , figures 2 and 3 . Serum protein profile in Kala-azar patients revealed three banding patterns (9-12) protein bands the (RM) values were ranged from (0.01-0.9). These differences in the number of bands and their molecular weight may be attributed to the difference in the clinical picture and severity of the disease figures 2 and 3.The first protein pattern demonstrates nine bands the RM values were ranged from (0.03-0.8), seven bands of these where different from the normal. These are bands number (1, 2, 3, 4, 6, 7, 8 and 9) with molecular weights (912.010, 707.945, 562.341, 501.187, 251.188, 158.489, 104.712, and 79.452) $\times 10^3$ Dalton respectively table 2 , figure 3. This band picture was found in 22 patients (36.66 %) table 3 .The second protein pattern shows twelve bands; the RM values were ranged from (0.01-0.7), of which nine where abnormal in particular, bands number (2, 3, 4, 5, 6, 9, 10,11 and 12) with a molecular weights (912.010, 891.250, 794.328, 562.341, 501.187, 251.188, 223.872, 112.201, 97.723) $\times 10^3$ Dalton respectively table 2 , figure 3. This profile was found in 30 patients (50 %) table 3.The third protein pattern shows ten bands; the RM values were ranged from (0.07-0.78), eight out of these were abnormal. These are bands number (1, 2, 3, 6, 7, 8, 9 and 10) with a molecular weights (794.328, 562.341, 501.177, 251.188, 199.526, 158.489, 97.723 and 70.794) $\times 10^3$ Dalton respectively table 2, figure 3. This profile was found in 8 patients (13.33%) table 3.

Table 2 : RM values and molecular weight for each band in different protein profile groups.

Band No.	Control RM	Control MW$\times 10^3$ Dalton	G1-RM	G1-MW$\times 10^3$ Dalton	G2-RM	G2-MW$\times 10^3$ Dalton	G3-RM	G3-MW$\times 10^3$ Dalton
1	0.01	1047.128	0.03	912.010	0.01	1047.128	0.07	794.328
2	0.1	707.945	0.12	707.945	0.03	912.010	0.2	562.341
3	0.35	331.131	0.2	562.341	0.05	891.250	0.26	501.177
4	0.41	275.422	0.26	501.187	0.07	794.328	0.35	331.131
5	0.72	104.712	0.41	275.422	0.2	562.341	0.41	275.422
6			0.47	251.188	0.26	501.187	0.47	251.188
7			0.6	158.489	0.35	331.131	0.54	199.526
8			0.72	104.712	0.41	275.422	0.6	158.489
9			0.8	79.452	0.47	251.188	0.74	97.723
10					0.49	223.872	0.78	70.794
11					0.6	158.489		
12					0.74	97.723		

Table 3 : Different protein profiles with the percentage of patients in each group

Group	Total No. bands	No. abnormal bands	No.patients	% patient
1	9	7	22	36.66
2	12	9	30	50
3	10	8	8	13.33

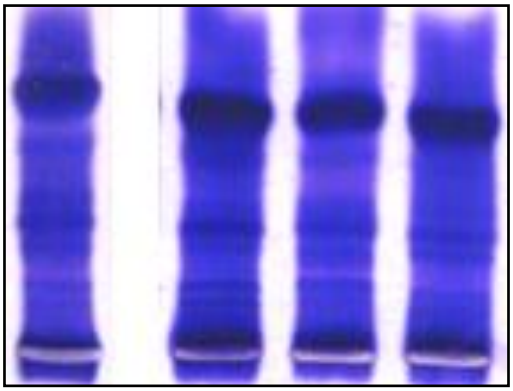

Figure 2 : Electrophoretic patterns obtained from Kala-azar patients sera and control non infected persons

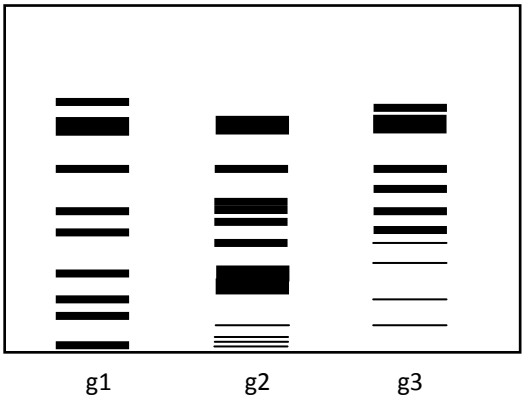

Figure 3 : Zymograph of different banding patterns of serum protein electrophoresis obtained from kala-azar patients.

Discussion

Visceral leishmaniasis is a potentially fatal disease caused by an intracellular protozoan parasite of the *Leishmania donovani* complex [(21)]. For successful control of the disease, efficient and reliable diagnosis is recommended [(22)]. Clinical presentations of spleenomegally [(23)], hepatomegally, fever, pallor [(24)], demonstration of the parasites in bone marrow aspirates or needle biopsy specimens of the spleen and lymph node or by *in vitro* cultivation are the definitive methods of diagnosis of visceral leishmaniasis [(25)].However, these methods are insufficiently sensitive, and the techniques are invasive, painful, and even hazardous [(26)] and need skilled personnel and equipped hospitals. Moreover, visceral leishmaniasis is occurring in places where health services are poorly developed. Poor socioeconomic conditions are associated with a higher risk of infection [(22)]. A number of serological tests have been developed and evaluated for the diagnosis of visceral leishmaniasis, including immunoflourescent-antibody tests IFAT [(27)], enzyme linked immunosorbent assay (ELISA) [(28)], dot ELISA [(29)], immunoblot analysis [(30)], and direct agglutination test (DAT) [(31)].Since these methods are some what complicated and required highly efficient personnel and very well equipped laboratories to be performed, making the need for easier and faster methods mandatory especially in endemic areas.The presence of *Leishmania donovani* soluble antigen(s), corresponding antibody and complement components in the serum of patient with active Kala-azar have been shown by Durate *et al*. [(32)] in human and in cotton rats infected with *Leishmania donovani* [(33)].Sera of normal subjects (control) showed the typical five bands. While sera of the diseased persons showed a three protein patterns, each pattern differ from the control in the number of abnormal bands -see the above results, figure2.The production of different protein bands with variable molecular weights in patients with active Kala-azar compared with normal (control) subjects may be attributable to the escalation of the host defense mechanism(s) against leishmania parasites. Higher levels of all IgG subclasses 1,2,3,4, as well as IgM have been reported during Kala-azar infection [(34,35)].Furthermore, the presence of three different protein profiles in this study among the tested patients with active Kala-azar may be related to different clinical presentation in young children with Kala-azar like the duration of fever, spleenomegally, hepatomegally, jaundice and response to therapy in Iraq [(36, 37, 38)] and other endemic countries like Sudan [(39)], India [(40)] and Iran [(41)]. On the other hand, all the three groups showed similarity at RM values of 0.2 and 0.26, o.47 and 0.6 with a MW=(562.341, 501.187, 251.188, 158.489)$\times 10^3$ Daltons respectively. Group 1 and 2 showed similarity at RM 0.03

with a MW 912.010×10^3 Daltons. While group 2 and 3 showed similarity at RM 0.07 and 0.74 with a MW (794.328 and 97.723) $\times10^3$ Daltons.

Recommendations

These similarities among the different groups may require further investigation in the search of a certain protein band that might be used as a marker for this disease. In addition, further studies may be required to identify the similarity in the bands between two different protein profile groups that may be attributable to the difference in the clinical presentation of the disease.

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