Lactate dehydrogenase (LD), alkaline phosphatase (ALP) isoenzymatic patterns in Iraqi children with visceral leishmaniasis before and after treatment with stibogluconate

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Abstract The mean levels of alkaline phosphatase (ALP), lactate dehydrogenase enzymes exhibited a significant elevation in visceral leishmaniasis (VL) patients compared to the control. There was no significant change in relation to the sex and age. ALP isoenzymes revealed three banding patterns which differ from the three zymodemes which were obtained from control group. These differences may be due to isoenzymes activity of patients with VL before and after therapy. Lactate dehydrogenase (LD) isoenzymes revealed five banding patterns differ from the five normal zymodemes. These differences mainly occurred due to LD isoenzymes activity in patients with VL before and after therapy.

Keywords Lactate dehydrogenase (LD) · Alkaline phosphatase (ALP) · Visceral leishmaniasis (VL) · Isoenzymes · Isoenzymatic patterns · Zymodemes

Introduction

Visceral leishmaniasis (VL), also known as Kala-Azar, is a disseminated protozoal infection caused principally by *Leishmania donovani* and *Leishmania infantum* (synonym

Leishmania chagasi in South America). Occasionally (van Griensven & Diro 2012). The worldwide prevalence of VL is estimated to be 12 million cases, with approximately 0.2–0.4 million new VL cases each year. More than 90 % of global VL cases occur in just six countries: India, Bangladesh, Sudan, South Sudan, Brazil and Ethiopia (Alvar et al. 2012). Several studies have reported derangement in the level of some enzymes during VL such as ALP (Mathur et al. 2008) and lactate dehydrogenase (Al-Saffar and Al-Mudhaffer 1979).

Enzymes

Enzymes are specific biologic proteins that catalyze biochemical reactions without altering the equilibrium point of the reaction or being consumed or undergoing changes in composition (Bishope et al. 2000). Without enzymes, life cannot exist and they are the tools that create life (Marshall 2000). More than 3,000 different enzymes have been identified in the human body. They build new proteins, cells, tissues, and organs, as well as break down diseased tissues. They are found in all tissues and they are present in much higher concentration inside than outside the cell (Benyon 1998).

Enzymes are released into the systemic circulation as a result of:

- (1) Increased rate of cell turnover during active growth or tissue repair or cancer (Schwartz 1982).
- (2) Necrosis or severe damage to cells (Lee & Goldman 1986).
- (3) Induction by disease or drugs (Zeltzer et al. 1986).
- (4) Biliary or pancreatic obstruction, as well as renal insufficiency also causes increased enzymes concentration (Moss & Henderson 1999).

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In most disease states, in which enzyme elevations occur, the cause is increased membrane permeability, usually secondary to cell injury or necrosis. Occasionally, increased enzyme levels in serum are caused by increased rates of intracellular synthesis and the subsequent diffusion of these secreted enzymes into the circulation (Pincus and Schaffner 1996).

Alkaline phosphatase (ALP)

Alkaline phosphatase, ALP (EC.3.1.3.1) belong to a group of enzymes that catalyze the hydrolysis of a wide variety of phosphomonoesters at an alkaline pH (Rodwell 1981). It is a non specific enzyme capable of reacting with many different substrates (Bishope et al. 2000).

ALP is present in practically all tissues of the body, especially at or in the membrane. It is present at particularly high levels in intestinal epithelium, kidney tubules, bone, liver and placenta (McComb et al. 1979). The enzyme requires some divalent ions such as Mg^{+2} , Co^{+2} and Mn^{+2} as activators of the enzyme while Cu^{+2} , Hg^{+2} , Zn^{+2} , Ca^{+2} , phosphate, borate, oxalate and cyanide are inhibitors of all forms of the enzyme (Varley et al. 1980). Although ALP displays considerable inter-or -intra tissue heterogeneity, rarely are there more than 2 or 3 forms found in any one serum sample.

The forms present in sera of normal adults probably originate mainly from the liver, with up to half of the total activity coming from the skeleton; these two forms of ALP are markedly age-dependent. A small amount of intestinal ALP may also be present (Raymond et al. 1991).

Electrophoresis is considered the most useful single technique for ALP isoenzymes analysis. The liver fraction migrates the fastest followed by bone, placenta and intestinal fraction, there exist some abnormal fractions that are associated with neoplasms (Posen & Doherty 1981).

Smith et al. (2000) listed the physiological conditions that are associated with increased plasma ALP activity, which are:

- (1) Normal pregnancy as a result of release of ALP from the placenta which may cause its total activity in

placenta to rise within second and third trimester of about twice normal adult levels.

- (2) Infancy and childhood, the upper reference values here being as much as 3–4 times the adult values. This increase is a direct result of the intestine osteoplastic activity associated with normal bone growth.
- (3) Meals containing fat which cause transient small increase in plasma intestinal ALP activity.

The bulk of ALP in the serum of normal patients is made up of liver and bone ALP. The normal ALP values in the plasma are 30–85 IU/l in the adult (Martin 1981). Elevated levels of ALP are seen in a variety of bone disorders (Warren 1981).

Variety of malignancies including ovarian, lung, and gastrointestinal cancers, were found to have elevated levels of ALP enzyme, such as leishmaniasis (Yousif 1981) and schistosomiasis (Juma and AL-Jeboori 1999).

ALP levels are significantly decreased in the inherited condition of hypophosphatasia. Subnormal activity is due to the absence of the bone isoenzyme and results in adequate bone calcification (Bishope et al. 2000).

The precise metabolic function of the enzyme is not understood, it appears that the enzyme is associated with lipid transport in the intestine and with the calcification process in bone (Smith et al. 2000).

Measurement of ALP was usually done by using enzymatic colorimetric methods, through the use of buffers and chromogenic substrate in commercial kits, used for this purpose (McComb et al. 1979; Varley et al. 1980). Immunoassay had also been used for bone ALP (Broyles et al. 1998) and immunofluorescence techniques (Shibano et al. 1999).

Lactate dehydrogenase (LD)

Lactate dehydrogenase (EC.1.1.1.27) is cytoplasmic enzyme that transfers H^+ and plays a role during the anaerobic glycolytic pathway. LD catalyzes the oxidation of Lactate to pyruvate in the presence of nicotinamide adenine dinucleotide (NAD) as hydrogen acceptor (Bishope et al. 2000) as follows:.

The enzymatic activity of LD is proportional to the rate of production of NADH (McComb 1983; Alacam et al. 1996). This enzyme is widely distributed in the body. Very high activities are found in the heart, liver, skeletal muscle, kidney and erythrocytes. It is present in lesser amount in the lung, smooth muscle and brain (Bruns et al. 1981).

LD is released to peripheral blood in case of excess heart or cold, starvation, dehydration and injury expose to bacterial toxins. The serum LD is highly sensitive but not specific test and this could be enhanced by isoenzymes analysis (Lott & Nemensanzsky 1987).

The enzyme has a molecular weight of 128,000 daltons, and is composed of four polypeptide chains with molecular weight of 32,000 daltons each.

Two different polypeptide chains, designated H (heart) and M (muscle), combined in five arrangements to yield the five major isoenzymes each under separate genetic control (Mackawa & Kanno, 1989). The five major fractions each comprise four subunits, which are LD-1 (H₄), LD-2 (H₃M), LD-3 (H₂M₂), LD-4 (HM₃) and LD-5 (M₄). LD-6 had been identified in the sera of severely ill patients its identify remain uncertain (Moss & Henderson 1999). LD-1 migrates most quickly towards the anode, followed in sequence by the other fractions with LD-5 migrating the slowest (Mercer 1978).

Enzyme value may be moderately elevated in myocarditis and cardiac infarction with hepatic congestion, sever shock and in anorexia (Lee & Goldman 1986). In malignancies, LD is one of the enzyme systems preferentially produced and retained by cancer cells, being necessary to maintain tumor growth (Rotenberg et al. 1988).

The highest levels of LD were seen in pernicious anemia and hemolytic disorders. Liver disorders, such as viral hepatitis and cirrhosis, show slight elevations of two or three times (Bishop et al. 2000).

In parasitic disease, elevated serum LD levels have been reported in patients with Sarcocystis (Prass and Fayer 1981), toxoplasmosis (Sacks et al. 1983) and leishmaniasis (Akrawi 1985).

By measuring NADH consumption, this method was used to measure total LD activity (Moss & Henderson 1999). Several methods have been developed to study isoenzymes pattern of LD, these include: Electrophoresis, this procedure is most widely used, after electrophoretic separation, the isoenzymes can be detected either fluorometrically or colorimetrically, or by chemical inhibition methods, ion exchange chromatography and immunoprecipitations (Meckenzie and Henderson 1983; Paz et al. 1990).

Materials and methods

This study was conducted from May, 2003 to December, 2005. Follow up was done in Medical Research Center/Al-

Nahrain Medicine College and Central Public Health Laboratory/Baghdad.

Subject selection

Blood samples were collected from 86 children under 6 years old in Al-Zahraa Children and Maternity Hospital/ Najaf and Babil Maternity and Children Hospital, AL-Khadhmiya Teaching Hospital, Central Children Hospital, Al-Ilwia Children Hospital, AL-Mansoor Children Hospital and Central Public Health Laboratory.

Subjects were divided into two groups:

- Patients (confirmed VL group)* fifty two patients with splenomegaly, positive (microscopic examination or culture) bone marrow aspirate and positive dipstick assay for VL. These samples were used as confirmed samples in this study.
- Healthy control* thirty-six blood samples were collected from children under six years old from different primary health centers with no history of living in endemic areas with VL and who were apparently healthy by physical examination.

Sample collection

Five millilitre blood was withdrawn from each patient and control collected in a plain tube from 52 children with disease before and after administration of sodium stibogluconate injection (28 days of sodium stibogluconate therapy). Each blood sample obtained in a plain tube allowed to clot, the clot was removed by gentle swirling with a wood stick centrifuged as soon as possible and serum separated, liquated into portions to avoid repeat freezing and thawing then stored at −20 °C until use.

Parasitological diagnosis

Bone marrow samples (biopsies) were aspirated from children who were suspected of being infected with VL by specialists. The patient was usually antisepticized locally with iodine followed by spirit. A puncture was made by means of a dry sterile bone marrow needle. About 1 ml of bone marrow was aspirated; portion of the aspirate were introduced aseptically into different culture media, the process being carried out to a flame on a clean sterile table in a room where the door and windows were kept closed during the operation to eliminate any possible source of draft; talking was not allowed. All these precautions were taken so that possible sources of contamination were cut down to the lowest level. (Rassam and AL-Mudhaffar 1979).

Bone-marrow sample was smeared on to a clean slide, and then air dried and fixed with methyl alcohol for 1–2 min. The slide was stained with 10–15 % Giemsa stain for 30 min, washed in tap water and air dried. The slides were examined microscopically under oil-immersion lens for LD bodies, the smears were considered negative for LD if these bodies were not seen after examination.

Alkaline phosphatase (ALP) activity assay

The activity was determined in the serum and the assay was carried out using bioMerieux phosphatase alkaline-kit, according to the following reaction:

The phenol liberated was measured in the presence of amino-4-antipyrine and potassium ferricyanide. The presence of sodium arsenate in the reagent stopped the enzyme reaction.

(a) Reagents

Reagent 1: Substrate buffer, which contains:	
Dinatrium phenylphosphate	5 mmol/l
Carbonate—bicarbonate buffer pH10	50 mmol/l
Reagent 2: Standard solution, which is made of:	
Phenol equal to 20 kind and king unit	
Reagent 3: Inhibitor, which is composed of:	
Amino-4-antipyrine	60 mmol/l
Sodium arsenate	75 gl/l
Reagent 4: Color reagent, which is made of:	
Potassium ferricyanide 150 mmol/l	150 mmol/l

(b) Procedure

Set up the following tubes according to Table 1.

- Mix, let stand for 10 min in the dark.
- Measure at wave length 510 nm (Hg 492); for zero adjustment we used reagent blank.

(c) Analysis of samples

The activity was determined by the following equation:

$$\text{Calculation} = \frac{\text{OD serum sample} - \text{OD serum blank}}{\text{OD standard}} \times n$$

where OD is the optical density, n: 142 u/l.

Table 1 Experimental design for ALP estimation in VL patients

	Tested sample	Serum blank	Standard	Reagent blank
Reagent 1	2 ml	2 ml	2 ml	2 ml
<i>Incubate for 5 min at 37 °C</i>				
Serum	50 µl	–	–	–
Reagent 2	–	–	50 µl	–
<i>Incubate for exactly 15 min at 37 °C</i>				
Reagent 3	0.5 ml	0.5 ml	0.5 ml	0.5 ml
<i>Mix well</i>				
Reagent 4	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Tested samples	–	50 µl	–	–
Distilled water	–	–	–	50 µl

Lactate dehydrogenase (LD) activity assay

The activity was determined in the serum; the assay was carried out using RANDOX kit for colorimetric determination of LD.

This determination was based on the reduction of pyruvate to lactate in the presence of NADH by the action of LD :

The pyruvate that remained unchanged reacted with 2,4-dinitrophenylhydrazine to give the corresponding phenylhydrazone which was determined colorimetrically in an alkaline medium.

(a) Reagents

(1) Buffer which is made of:	
Phosphate buffer (pH 7.45)	100 mmol/l
Sodium pyruvate	0.8 mmol/l
(2) NADH	
	1 mg/ml
(3) Color reagent, which is made of	
2,4- Dinitrophenylhydrazine	1 mmol/l
HCL	1 mmol/l
(4) NaOH	
	1.6 % (0.4 N)

The specimen (serum) should be freshly collected and free from hemolysis.

(b) Procedure

Pipette in cuvette according to Table 2.

- Mix, allow standard for 10 min.
- Read absorbance of samples against distilled water at wave length 520 nm.

Table 2 Experimental design for LD estimation in VL patients

0.9 ml	NADH
Place in a water bath at 37 °C for 2-3 min.	
0.1 ml	Sample
Place in a water bath at 37 °C for exactly 30 min.	
1.0 ml	Colour reagent
Mix, allow to stand at room temp. for 20 min.	
10 ml	Sodium hydroxide

Fig. 1 Calibration curve for lactate dehydrogenase enzyme

- Enzyme activity was determined by reference to the calibration curve, the corresponding values in U/l.

(c) Calibration curve

Absorbance	0.50	0.42	0.33	0.28	0.19	0.11
u/l	0	111.4	223.1	334.9	390.6	446.3

Calculation of values was done by interpolation with calibration curve Fig. 1.

Isoenzymes of Alkaline phosphatase (ALP)

ALP isoenzymes were developed after electrophoresis as described by Rassam et al. (1996).

* Solutions for developing ALP isoenzymes :

0.01 g	Nitroblue trizolium (NBT)
0.005 g	5-bromo-4-chloro-3-indolyl phosphate
0.07 g	MgCl ₂
20.0 ml	Tris glycine buffer

Kept in 40 °C till bands appeared.

Isoenzymes of lactate dehydrogenase (LD)

* Solutions for developing LD isoenzymes :

10 ml	Tris glycine buffer
5 ml	Sodium lactate
3 ml	PMS
3 ml	NBT
0.05 g	NAD

Kept in 40 °C till bands appeared.

Results

Alkaline phosphatase (ALP)

A highly significant increase ($p < 0.05$) in the levels of VL patients was seen, compared to that of the normal healthy control group (177.17 ± 7.68 versus 80.45 ± 6.50 u/l) Table 3. The ALP activity was also assessed in relation to some clinical parameters (sex and age) and there were no significant changes in its value ($p > 0.05$) Table 4.

Lactate dehydrogenase (LD)

There was highly significant increase ($p < 0.05$) in serum LD mean level of VL patients compared to the healthy control group (341.88 ± 9.62 versus 144.18 ± 7.75 u/l) (Table 3). There were no significant changes in relation to some clinical parameters (sex and age) in its value ($p > 0.05$) (Table 4).

Isoenzymes

Isoenzymes of alkaline phosphatase (ALP)

There are three banding patterns for ALP enzyme activity which were discerned among the sera of 86 patients, that

Table 3 Mean concentration $\pm$ SE of ALP, LD enzymes in the sera of 52 visceral leishmaniasis patients & healthy control

Enzymes (u/l)	Control	Patients	<i>p</i> value
ALP	80.45 ± 6.50	177.71 ± 7.68	<0.05
LD	144.18 ± 7.75	341.88 ± 9.62	<0.05

Table 4 Mean concentration $\pm$ SE of ALP, LD enzymes in the sera of 52 VL patients in relation to sex & age

Enzymes (u/l)	Sex		<i>p</i> value	Age (year)		<i>p</i> value
	Male	Female		<Year	>Year	
ALP	81.2 $\pm$ 4.3	80.5 $\pm$ 3.2	>0.05	83.3 $\pm$ 3.8	75.6 $\pm$ 2.1	>0.05
LD	146.7 $\pm$ 3.5	145.2 $\pm$ 4.8	>0.05	155.2 $\pm$ 4.6	135.3 $\pm$ 3.4	>0.05

Fig. 2 photograph of the electrophoretic patterns obtained with kala-azar disease for alkaline phosphatase (ALP) isoenzymes. *Strip 1* control sera (healthy children). *Strip 2* kala-azaric sera *Strip 3* sera from children after treatment **Fig. 3** Zymograph of ALP (alkaline phosphatase) isoenzymes obtained with kala-azar disease. *Strip 1* control serum. *Strip 2* kala-azaric serum. *Strip 3* sera from children after treatment

differ from three zymodemes which were obtained from 18 healthy control, these differences due to isoenzymes activity of patients with VL before and after therapy Figs. 2 and 3.

(a) Control group

Alkaline phosphatase enzyme of 18 healthy control sera (100 %) were separated into 3 major fractions; liver, bone and intestine. Liver ALP normally runs as an α -2 band, bone runs in the α -2/ β -region and intestinal isoenzyme was usually found in the β -2/ γ -region Figs. 2 and 3.

Fig. 4 Photograph of the electrophoretic patterns obtained with kala-azar disease sera for lactate dehydrogenase (LD) isozymes. *Strip 1* sera from healthy children. *Strip 2* sera from infected children. *Strip 3* sera from children after treatment

(b) ALP of VL patients before treatment

Forty patients (100 %) had different bands (with excess amount) between α -2 and β -region towards the normal liver band, in addition to the presence of a thick band of skeletal isoenzyme. The intestinal band had a thin band in comparison with control group Figs. 2 and 3.

(c) ALP of VL patients after treatment

Forty six patients (100 %) showed a thin liver band of ALP enzyme, but relatively thicker than that of skeletal and intestinal isoenzyme band Figs. 2 and 3.

Isoenzymes of lactate dehydrogenase (LD)

There are five banding patterns for LD isoenzyme activity in VL patients differ from the control group. These differences mainly occurred due to the ALP isoenzyme activity in patients with VL before and after therapy Figs. 4 and 5.

(a) Control group

Twenty children (100 %) of normal healthy control group, sera LD enzyme was separated into five major

Fig. 5 Zymograph of LD (lactate dehydrogenase) isozymes, obtained with kala-azar disease. *Strip 1* control sera. *Strip 2* kala-azar sera. *Strip 3* kala-azar sera after treatment

fractions (LD1, LD2, LD3, LD4 and LD5), LD-1 migrates most quickly towards the anode, followed in sequence by the other fractions, with LD-5 migrating the lowest. All the LD isoenzymes showed the greatest amount (thick bands) except LD4 characterized by a decrease in amount (faint band) Figs. 4 and 5.

(b) LD of VL patients before treatment

Thirty five patients (85 %) showed a similar LD banding pattern to the control group, with the greatest amount (thick bands) in LD4 and LD5. The other fractions (LD1, LD2 and LD3) had a diminished quantities (thin bands) in the order : LD2, LD1 and LD3 Figs. 4 and 5.

(c) LD of VL patients after treatment

Twenty patients (83 %) showed similar banding patterns to the control group with two clear thin bands of LD1 and LD2. The other fractions had a slight elevated quantity (but, thin bands) in comparison with LD4 and LD5 isoenzymes Figs. 4 and 5.

Discussion

Alkaline phosphatase (ALP)

The activity of ALP was found to be significantly higher in patients with leishmaniasis than in health control. ALP enzyme was found in many tissues, but highest concentration were found in biliary tract epithelium and kupffer's cells of the liver (Pagana and Pagana 1998). Some reports showed that any factor affecting the liver metabolism was found to cause an increase in ALP activity (Pincus & Schaffner 1996). The infancy and childhood showed high

ALP values as much as 3–4 times that of adult value (Smith et al. 2000). A damage or injury which was caused by VL liver tissue (principally parenchymal cells) led to releasing ALP into the blood circulation (Norbert and Tietz 1988).

Lactate dehydrogenase (LD)

Similar results also were obtained with LD enzyme. There was an increase in its activity in VL patients in comparison with healthy controls. In general this enzyme is usually used for detection of hepatocellular damage, whenever liver cells are damaged or injured, the liver enzymes leak out into the circulation across the damaged cell membrane (Moss et al. 1986). As we mentioned previously ADA play a role in the liver function (Bollinger et al. 1996), so its deficiency lead to liver disturbance, hence, elevated levels of LD in VL disease patients will be attributed to hepatic involvement by diseases (Kreisbergs 1978), as well as the rise in enzyme levels in blood may be due to tissue damage. This will lead us to understand the cause of LD elevation levels in liver VL patients.

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