ORIGINAL ARTICLE

UTILITY OF SERUM LDH ISOFORMS IN THE ASSESSMENT OF MYCOBACTERIUM TUBERCULOSIS INDUCED PATHOLOGY IN TB PATIENTS OF SAHARIYA TRIBE

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ABSTRACT

The present study was carried out in the Sahariya tribe of Central India, which reportedly have high prevalence of pulmonary tuberculosis. Total serum LDH and its tissue specific isoforms were estimated in TB patients and matched healthy controls to test the utility of LDH as diagnostic marker for tuberculosis. About 210 sputum positive cases and 328 age and sex matched sputum negative controls were recruited. The spectrophotometric and densitometric analysis of each LDH isoform was carried out in both cases and controls. The mean values of serum LDH were estimated and compared for each class by t-test. The statistical comparisons were made between sputum negative controls and sputum positive cases by Mann-Whitney's U test. The spectrophotometric estimation of serum LDH revealed significant ($P=0.0016$) increase in its level in cases (290 IU/L) as compared to controls (248 IU/L). The densitometric analysis of individual LDH isoforms in cases and controls demonstrated significant elevation in LDH1 ($P>0.05$), LDH2 ($P>0.05$) and LDH3 ($P<0.005$) in sputum positive cases in comparison to sputum negative controls. Our study revealed a positive correlation between serum LDH level and the presence of mycobacteria and their load, suggesting utility of LDH as an important diagnostic marker of tuberculosis induced stress, at least in tribal areas lacking access to modern clinical tests.

KEY WORDS

LDH isoforms, Tuberculosis, Sahariya tribe, Acid Fast Bacilli.

INTRODUCTION

Lactate dehydrogenase (LDH) is an intracellular enzyme found in various human tissues such as skeletal muscle, heart, liver and kidneys. It is basically a hydrogen transfer enzyme that catalyses the oxidation of L-lactate to pyruvate using nicotinamide adenine dinucleotide (NAD)⁺ as hydrogen

acceptor, the final step in the metabolic chain of anaerobic glycolysis. LDH is composed of two subunits (H and M), which assemble to form the tetrameric protein. The various qualitative associations of these subunits result in the formation of five different LDH molecules, called 'isoenzymes' or 'isoforms' (i.e. LDH1, LDH2, LDH3, LDH4 and LDH5) that can be separated as five distinct bands by non-denaturing (native) polyacrylamide gel electrophoresis. The LDH1 isoform (H₄), found in heart muscle, is composed of four H subunits and is the lightest one. On the other hand, LDH5 (M₄) is found in skeletal muscle and liver and is the heaviest of all the isoforms, composed of four M subunits. LDH2 (3H1M), LDH3 (2H2M) and LDH4 (1H3M) show intermediate mobilities, and are found in varying degrees in many tissues.

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LDH as a pathophysiological marker has been studied in relation to several opportunistic infections, including infection by *Pneumocystis carinii pneumonia* (PCP), tuberculous and bacterial pneumonia (1, 2). Its level is increased in patients with megaloblastic anaemia, acute leukemia, urinary tract infection, pulmonary embolism, pulmonary tuberculosis in adults, in tubercular & cerebrospinal meningitis (3-7) and more recently in testicular tuberculosis (8), reflecting its diagnostic utility in specific clinical cases. It may also serve as a sensitive marker of epidermal (skin) toxicity (9) or stress induced by some other pathological conditions (10, 11) as also shown in case of broncho alveolar lavage fluid (BALF) (12-14). More recently, serum LDH level has been proposed as predictor of acute-graft-versus-host disease after allergenic transplantation (15) and survival in case of osteosarcoma (16). The role of LDH-A has also been implicated in breast cancer development (17).

Thus, the quantitative assay and electrophoretic profiling of LDH isozymes in a biological sample can be used as a marker for the diagnosis of infectitious diseases and other pathological conditions affecting multiple organs like Liver, Heart, Lungs, etc.

The present study was carried out in Sahariya tribe, a primitive tribal group of Central India, living under poor socio-economic conditions and having high incidence and prevalence of pulmonary tuberculosis, especially in young age group (18, 19). The aim of this study was to test the hypothesis that extracellular or serum levels of LDH and its isoforms are related to specific cell or tissue damage and may, thus, indicate the tissue source of the LDH release into blood serum. Further, it was thought to see, if the level of LDH or any of its isoforms is correlated with the bacterial load in the sputum of TB patients. Our investigation has confirmed further the significance of both total LDH and its isoforms to be used as indicators of the severity of the disease and as marker for identification of tissue or organ affected.

MATERIALS AND METHODS

In this study, 320 sputum negative controls (SNC) and 209 sputum positive case (SPC) subjects were recruited. The sputum samples were collected in 30 ml sterile disposable plastic containers. The sputum smears were made on clean glass slides, dried and stained following Ziehl-Neelsen's method (20) in the field laboratory at district hospital, Sheopur, M. P. (India). The sensitivity of Ziehl-Neelsen's staining techniques is about 75%, yet the specificity is uniformly high, about 95-99%. Ziehl-Neelsen's method is commonly used for

sputum smear staining because of its simplicity, high sensitivity and specificity to detect acid fast bacilli. The sputum smears were assigned negative and positive status as per the standard RNTCP (Revised National Tuberculosis Control Programme) protocol (21, 22) based on mycobacterial counts per oil immersion field under a Nikon photomicroscope. All the sputum three positive, two positive, one positive and sputum-negative samples were properly recorded after smear examinations.

Blood sampling was carried out from SNC (n=320) and SPC (n=209) subjects with prior well-informed and written consent of the donor. The work plan and protocols of this study were approved by the Institutional Ethics Committee as well as by Health and Tribal Welfare Department, Government of Madhya Pradesh, India. About 5 ml blood was withdrawn from subjects (cases and controls) using sterile hypodermic syringes in the presence of an expert clinician or doctor and stored in blood collecting vials containing EDTA under optimum conditions. The blood samples were stored on ice and brought back to the laboratory. Each blood sample was centrifuged at 447xg for 10 min and the serum was transferred to separate sterile vials and stored at -20°C.

The total LDH content in each serum sample was estimated following the method of Bach *et al* (23) with minor modifications. Fifty microliters (50 µl) of serum sample was incubated with 80 µl of freshly prepared NADH (1 mg/ml) and 0.75 mM/l pyruvate in phosphate buffer (pH 7.5) at 37 °C for 30 min, followed by addition of 80 µl of 0.2 mg/ml 2, 4, dinitrophenyl hydrazine (DNPH). The reaction was stopped by adding 40 µl of 3.5 mol/l NaOH and the absorbance was measured at 450 nm. After quantitative estimation of total LDH in the serum, total LDH content per micro litre of serum was calculated.

Equal amount of LDH (50U) from each sample was loaded on to 6% native PAGE to resolve all the five isoforms of the lactate dehydrogenase in all the case as well as control serum samples. After electrophoretic separation, the gel was processed for LDH specific staining. The gel was soaked in a glass petri plate containing LDH staining dye (10 mg NAD, 10 mg NBT, 1 mg PMS and 5 ml of Sodium lactate as substrate), and incubated at 37°C for 10-15 min till LDH bands started appearing. Once, all the bands became clearly visible, the reaction was stopped by adding tap water and the gel was fixed and stored in 7% acetic acid.

The densitometric analysis of all five LDH isoforms (LDH1, LDH2, LDH3, LDH4 and LDH5) was carried out in only few representative samples from both sputum negative controls

(n=63) and sputum 1+ (n=10), sputum 2+ (n=12) and sputum 3+ (n=22) cases using UVItrek (UK) gel doc system provided with UVIband software. For densitometric analysis, only those samples were chosen in which all the five LDH isoform bands were distinctly visible. The adjacent blank lane was taken as reference for analysis of each lane. The percentage volume covered by each band in each lane was measured and further analysed for intra (among controls) and inter-group (control vs. case) comparisons.

The p values were calculated by comparing means of each class and applying paired t-test (two tailed), followed by Bonferroni corrections. The concentrations of LDH (IU) are presented as mean, median and as 50th and 75th percentiles. The statistical comparisons between sputum-negative and sputum-positive groups were made using Mann-Whitney's U test. P value of 0.05 was considered statistically significant.

RESULTS

Based on the number of mycobacterial counts per oil immersion field per slide, smears were divided into three distinct categories as, sputum negative with no mycobacterium seen at all, sputum one positive (+ve) with 10-99 AFB (Acid Fast Bacilli) per 100 oil immersion field, sputum two positive (++) with 1-10 mycobacteria per 50 oil immersion field (counted in 50 fields per slide) and sputum three positive

(+++ve) with 10 or more mycobacteria per oil immersion field (counted in 20 fields per slide). Out of 209 cases (SPCs) 24 individuals were recorded as sputum 1+ve, 51 as 2+ve and 134 in the category of 3+ve.

Serum LDH level: A comparison of total serum LDH content between SNCs and SPCs revealed its level to be significantly high ($P < 0.1$) in SPCs than in SNCs (Table, Fig 1). The sputum positive (1+ , 2+ and 3+) subjects showed elevated level of LDH, especially in sputum three positive subjects, which revealed significantly ($P = 0.004$) high LDH content (Table). The mean, median, 50th and 70th percentile values of LDH level were calculated in sputum negative as well as sputum positive subjects for comparative analysis. To make non-parametric comparisons of LDH level in case and control groups, Mann-Whitney analysis was performed (Table). **LDH staining:** The blood serum was collected from sputum negative healthy subjects as well as from sputum positive (AFB positive) TB patients, and run on 6% non-denaturing gel (native PAGE) to resolve all the five LDH isoforms. It was observed that in most of the sputum positive samples (sputum 1+ve, 2+ve and 3+ve, Fig 2, lanes 7/8, 3, and 6, respectively), TB patients had elevated level based on visible staining intensity of LDH1, LDH2 and LDH3 isoforms as compared to that in sputum negative serum samples (Fig 2, lane numbers 2, 4, 5, 9, 10). Unexpectedly, certain sputum negative serum samples (Fig 2, lane numbers 1, 2, 5) also showed elevated level of LDH1,

Fig 1: Serum level of LDH (IU/L) in sputum negative (a) and sputum positive (b) subjects

Table: Comparison of total serum LDH content (A-D) and its isoforms (E) in sputum positive cases and sputum negative controls of Sahariya tribe

Enzyme	Parameters	Sputum –ve (n= 320)	Sputum 1+ ve (n=24)	<i>P-value</i>	P-value after Boneferroni correction	
Serum LDH IU/L (A)	Mean	359.9	386.8	0.1273	0.06365*	
	Median	342.0	410.5			
	50th percentiles	342.0	410.5			
	70th percentiles	459.6	553.5			
	Mann-Whitney's Mean rank	170.26	202.3			
Enzyme	Parametr s	Sputum –ve (n= 320)	Sputum 2+ ve (n=51)	<i>P-value</i>	P-value after boneferroni correction	
Serum LDH IU/L (B)	Mean	359.9	402.2	0.1406	0.0703**	
	Median	342.0	348.8			
	50th percentiles	342.0	348.0			
	70th percentiles	459.6	497.5			
	Mann-Whitney's Mean rank	182.7	206.5			
Enzyme	Parametr s	Sputum –ve (n=320)	Sputum 3+ ve (n=134)	<i>P-value</i>	P-value after boneferroni correction	
Serum LDH IU/L (C)	Mean	359.9	404.5	0.0042	0.002***	
	Median	342.0	335.0			
	50th percentiles	342.0	335.0			
	70th percentiles	459.6	502.8			
	Mann-Whitney's Mean rank	216.1	254.7			
Enzyme	Parametr s	Sputum –ve (n=320)	All sputum positive (n=209)	<i>P-value</i>	P-value after boneferroni correction	
Serum LDH IU/L (D)	Mean	359.9	401.6	0.0016	0.0008****	
	Median	342.0	342.0			
	50th percentiles	342.0	342.0			
	70th percentiles	459.6	536.0			
	Mann-Whitney's mean rank	248.0	290.9			
Enzyme IU/L (E)	Normal range ^{\$}	Sputum negative (n=63)	1+ (n=10)	Sputum positive 2+ (n=12)	3+ (n=22)	P-value
LDH1%	14–28	25.20	22.40	29.70	23	>0.05
LDH2%	34–44	40.10	28	34.30	34.60	>0.05
LDH3%	14–23	19.40	35	18.70	21.80	<0.005
LDH4%	6–11	8.90	5.60	9.70	9.10	>0.05
LDH5%	7–18	6.10	8.80	7.20	11.20	>0.05

Bonferroni's corrections were applied. *P value (P= 0.06) and **P value (P=0.07) were NS (Non-Significant), *P value (P=0.002) was statistically significant after Bonferroni correction (for 2 combinations), ****P value (P=0.0008) was highly significant after Bonferroni's correction (for 2 combinations).

[§]Drent et al, 1999

LDH2 and LDH3), which probably may be due to some other undiagnosed infections, other than *Mycobacterium tuberculosis*.

Once all the five isoforms of LDH were resolved on the gel, the images were captured by gel documentation system for

further densitometric analyses. Each LDH band, representing individual isoform, was quantitated by UVItrek software to calculate the relative density in arbitrary unit in the respective sample. Each LDH isoform was quantitated in sputum negative as well as all the three categories of sputum positive samples, individually. Densitometric analysis of a representative gel

Fig 2: A. Five isoforms of LDH in serum samples of sputum negative and sputum positive Subjects of Sahariya tribe: Lane 1 & 2: Sputum negative, lane 3: Sputum 2+, lane 4 & 5: sputum negative, lane 6: Sputum 3+, lane 7 & 8: Sputum 1+ and lane 9 & 10: Sputum negative. **B.** Densitometric analysis of five LDH isoforms in sputum negative and sputum positive samples. Lines indicate a: LDH1; b: LDH2; c: LDH3; d: LDH4; e: LDH5

having sputum negative as well as sputum positive samples is shown in Fig 2. It is clearly evident from Fig 2B that LDH1, LDH2 and LDH3 levels are elevated in sputum 1+ve, 2+ve and 3+ve serum samples in lane number 7/8, 3 and 6, respectively. The lanes 7/8 (1+), however showed comparatively low expression of LDH2 and LDH3 than sputum 2+ (lane 3) and sputum 3+ (lane 6). The sputum 1+ grade is

given after observing 10–99 acid fast bacilli in 100 oil immersion fields, means very low count of tubercle bacilli. Therefore, the number of bacilli in sputum 1+ category may not be sufficient to reach a threshold, which is actually required to induce patho-physiological expression of serum LDH. Also, some time, due to manual contaminations during smear preparations, a negative slide may be given a status of 1+ (false positive), which may not reveal elevated level of any LDH isoform.

Densitometric analysis revealed that the level of LDH2 and LDH3 is highest in sputum 3+ve sample (Fig 2B, lane 6), while sputum negative serum has relatively low levels of LDH2 and LDH3 isoforms (Fig 2B, lane 4 and 5). The percentage of each isoform of LDH was also calculated in sputum negative and positive samples, which revealed that sputum positive serum samples, especially of 3+ve, has the highest percentage of LDH3, LDH4 and LDH5, while the sera of sputum 1+ve subjects showed LDH3 to be maximum. In contrast, sputum negative serum samples, showed the percentage of LDH2 to be highest. The percentage of other isoforms (LDH1, 3, 4, 5) in sputum negative sera were either equal or less than that of sputum positive serum samples (Table and Fig 2B).

To know, if LDH expression profile, in any way, is associated with the age-specific susceptibility to tuberculosis, which might validate its diagnostic importance, the patients were classified in different age groups (11-20 yrs, 21-30 yrs, 31-40 yrs, 41-50 yrs and 51-60 yrs) along with corresponding controls. The controls of age group 11-20 and 21-30 yrs showed relatively high level of LDH (397.2 IU/L and 412.4 IU/L respectively) in comparison to those of cases (350.9 IU/L and 410.6 IU/L, respectively). However, the older age groups (31-60 yrs) revealed LDH level to be significantly elevated ($P < 0.05$) in case subjects than in controls. The level of total serum LDH

Fig 3: A. Age group-wise expression of LDH in sputum negative controls and sputum positive case subjects of Sahariya tribe. **B.** Percentage of various LDH isoforms in the sputum negative controls and the three categories (1+, 2+, 3+) of sputum positive case subjects of Sahariya tribe

in various age groups and the levels of LDH isoforms in case and control categories are given in Fig 3 A & B, respectively.

DISCUSSION

The present study has further substantiated the evidences in support of diagnostic utility of LDH by demonstrating significant increase in the serum LDH level in sputum positive subjects as compared to sputum negative control subjects of the tribe, indicating a consequence of *Mycobacterial infection*, justifying the diagnostic importance of LDH. Unexpectedly, some of the sputum negative samples, particularly from children and adult subjects of age group 11-20 and 21-30 years, showed comparatively high level of serum LDH, which seems to reflect some other pathogenic infections or clinical complications resulted from severe malnutrition and poor immunity (data not shown) against various pathogens, commonly observed in this tribe (our unpublished data). The higher level of serum LDH in all other age groups among cases is more likely due to mycobacterial induced tissue injury as well as other pathophysiological conditions, which remained undiagnosed during this study. The results clearly indicate that adult and aged tribesmen have higher prevalence and susceptibility to mycobacterial infection as compared to children or young ones of the tribe.

It is clearly evident from the densitometric analysis of LDH isoforms in sputum negative and sputum positive subjects that sputum positivity for AFB, in general, and increasing mycobacterial load, in particular, may affect the level of individual LDH isoform in a TB patient, which indicates the clinical outcome or severity of the disease. In other words, the sputum positive clinical status may affect tissue/organ-specific expression of LDH. In this study, the sputum positivity or increasing mycobacterial load in Sahariya TB patients was found to affect the serum expression level of LDH2, LDH3 and LDH5, indirectly pointing to the existing pathological conditions of erythrocytes, lungs and liver/muscles, respectively. It is likely that the lung and liver/muscle isoforms (LDH3 and LDH5, respectively) might have been transported into serum following mycobacterial infection, at least, in the subjects under study (some time some other infection/s may also be involved).

Thus, serum LDH still remains as one of the important parameters to assess the cell/tissue specific stress or pathology, caused by *Mycobacterium tuberculosis*. The observed correlation between serum LDH level and sputum mycobacterial load alone or in combination with its individual isoforms, especially LDH3 and LDH5 can thus be used as an

index for diagnosis of tissue/organ affected by the disease (clinical). It also indicates the severe patho-physiological conditions of the lungs and liver of TB patients of this tribe with high bacilli load, hence, may act as useful follow-up marker to assess the recovery rate in-vivo (at the lung or liver cell level, in cases of pulmonary or extra-pulmonary TB, respectively) in patients on TB chemotherapy.

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