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Serum glutamine synthetase activity as biomarker for tuberculosis diagnosis and monitoring anti-tubercular drug therapy success

Dipak Kumar Chattopadhyay*

Deptarment of Biochemistry, College of Medicine & JNM Hospital, Kalyani
The West Bengal University of Health Sciences, Salt Lake, Kolkata-700 064, West Bengal, India

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Tuberculosis is a major public health problem in developing countries. It is one of the most widely spread human diseases globally. While the new diagnostics for tuberculosis (TB) have created greater interest, their full impact on global propaganda against tuberculosis is yet to be evaluated. Most of the TB cases are prevalent in the underdeveloped and developing regions of the world where the means to diagnose and to treat TB cases are limited. As a result, the infectious TB cases might remain undiagnosed or diagnosed late. So, the need of the hour is to launch a rapid but simple, inexpensive, sensitive and specific test to diagnose TB cases at the earliest. The abundant presence of glutamine synthetase (GS) in the culture filtrate of pathogenic mycobacteria has correlation with the occurrence of poly L-glutamate/glutamine component in the cell wall of these pathogenic mycobacteria but not in non-pathogenic mycobacteria. The GS seems to be stable in the infected host and also is present in host tissues and fluid. GS is demonstrated to be present in the serum of subjects suffering from pulmonary or extra-pulmonary tuberculosis; but not detectable at all in normal control and disease control (lung disease control) subjects. With anti-tubercular (A-TB) drug therapy in pulmonary and extra-pulmonary tubercular subjects, the serum GS levels fall significantly. To confirm that only the *Mycobacterium tuberculosis (M. tuberculosis)* GS is being assayed, the serum GS activity may be assayed in the presence of L-methionine-S, R-sulfoximine (MSO), a selective inhibitor of *M. tuberculosis* GS. The concentration of this inhibitor is so selected that it is sufficient to inhibit mycobacterial GS activity but not the human (mammalian) GS activity. It increases the specificity of the test. Thus, an ELISA or latex agglutination test might be inducted to assay the serum GS activity for the rapid and reliable detection of active or latent tubercular subjects and also detecting the presence of drug sensitivity or the emergence of resistance against A-TB drugs. Nevertheless, for rapid field study, the card assay technique might be implemented; where when a drop of serum from a suspected subject be placed on the substrate for GS; a definite colour change would mark the test positive.

The assay for serum GS requires only a sample of serum from the patient and takes only few minutes to perform. Therefore, the results will be available instantaneously when the patient is at the medical facility centre or even at home and this will curtail the number of visits to the medical facility centre. This is really advantageous in developing and underdeveloped countries where in many cases the patients do not have the capability of making multiple visits to the medical facility centre. The test procedure can also be used as an assay to monitor the response and success of anti-tubercular drug therapy. The procedure can very well indicate whether the treatment is successful or a change in the antibiotic drug therapy would be needed.

Keywords: γ -glutamyl hydroxamate-Fe^{+3} complex, L-methionine-S, Pulmonary and extra-pulmonary tuberculosis, R sulfoximine, Serum glutamine synthetase, Transfer reaction

Worldwide, tuberculosis is one of the 10 causes of death and the leading cause from a single infectious agent (above HIV/AIDS). In 2017, TB caused an estimated 1.3 million deaths among HIV-negative people. India bears the major burden (27%) of tubercular cases globally[1].

The traditional diagnostics for TB as well as the newer diagnostics have their limitations and drawbacks as mentioned below.

a) Chest X-ray-insensitive and non specific test.
b) Sputum microscopy- time consuming and having low sensitivity in HIV co-infected subjects.
c) Solid media culture- requires significant lab infrastructure and time upto 8 weeks.
d) Interferon gamma release assays-poor performance for active disease.
e) Fluorescence microscopy-requires specialized equipment
f) Liquid media culture-false positivity can occur due to cross-contamination in the lab.

*Correspondence:
E-mail: drpadhyay1976@rediffmail.com

g) Polymerase chain reaction- Shows a large degree of heterogeneity.
h) X pert MTB- has lower sensitivity in HIV-infected patients and is not recommended for treatment monitoring.
i) Line probe assay- expensive and the main use being for the rapid detection of drug resistant tuberculosis.

Though greater interest has been produced by the newer diagnostics for TB, their full impact on global propaganda against TB is yet to be evaluated[2].

Instead of the institution of the X pert MTB assay as a newer rapid tuberculosis diagnostic, the difference between approximate global estimate of incidence and new cases of notification is about 4.1 million persons. So, to improve the rate of case detection, biomarkers are urgently needed, which are rapid, highly specific and sensitive to diagnose pulmonary as well as extra-pulmonary tubercular subjects and also to indicate progression from latent infection to clinical disease, to indicate risk of reactivation after cure and also to provide accurate end points for drug and vaccine trials[3].

The enzyme glutamine synthetase (GS), a dodecamer of identical 53 kda, plays a major role in nitrogen metabolism by catalyzing the synthesis of L-glutamine from L-glutamate, ammonia and ATP. In *M. tuberculosis*, GlnA1 is an essential enzyme as *gln*A1 mutants are not viable *in vitro* or *in vivo* and the other *gln*A1 homologs in *M. tuberculosis* (*gln*A2-4) cannot replace the functional loss of *gln*A1[4,5]. The presence of glutamine synthetase in appreciable amount in the culture filtrate of pathogenic mycobacteria has been reported. The presence of a poly L-glutamate/glutamine component in the cell wall of pathogenic mycobacteria (but not in non-pathogenic mycobacteria) has a strong correlation with this fact[6,7].

The compound L-methionine-SR-sulfoximine (MSO) has inhibitory effect on the GS enzyme activity of mycobacterial origin including *M. tuberculosis* but it has very little effect on mammalian glutamine synthetase[8]. It is also reported that the stability of *M. tuberculosis* GS is fairly high in the culture medium[9].This higher stability might be largely responsible for their higher concentration in the extracellular medium of broth culture. This fact gives the strong indication that this enzyme (GS) has the strong possibility to be stable and persistent in the infected host tissues and fluids.

It was reported that treatment of TB subject with MSO inhibiting the formation of poly-L-glutamate/ glutamine cell wall structure, had shown to inhibit mycobacterial growth giving clear indication that the GS had played a great role in mycobacterial homeostasis[10].

The complete reaction catalysed by glutamine synthetase consists of actually three steps-the transfer of phosphate from ATP to glutamate, addition of ammonia to phosphorylated glutamate producing protonated glutamine and the removal of proton from glutamine by the leaving phosphate. This exothermic reaction (yielding an activation free energy of 21.5 kcal/M) might be an opening door for more pronounced studies for the development of new anti-tuberculosis therapies[11]. Thus, the presence of GS was expected as a biomarker in the serum of tubercular subjects specially before the institution of anti tubercular drugs and its level could have been used as a means for monitoring the success of treatment. By incorporating MSO, the selective inhibitor of pathogenic mycobacteria, as a control in serum GS assay; should allow enhanced specificity.

Materials

The study was conducted at B.S. Medical College & Hospital, Bankura-722102(WB), India. The study on blood samples obtained from human subjects was approved in writing by the Ethics Committee of B.S. Medical College & Hospital. Before collection of blood, the verbal consent was taken from the tubercular subjects as well as from the normal control and lung disease control subjects. The following groups of subjects were taken into consideration.

Normal control subjects

They were the healthy relatives of the patient with TB but without any clinical signs, symptoms, or X-ray findings suggestive of tuberculosis or any sort of diseases. Needless to say, they were sputum negative for acid fast bacilli.

Disease control subjects (lung disease control)

They were the patients suffering from respiratory tract infection (RTI) or bronchiectasis or bronchial asthma or bronchogenic carcinoma. In these subjects, there was no sign or symptom at all of tuberculosis. They were selected from the patients attending the Out Patient Department of B.S. Medical College & Hospital, Bankura (WB).

Tubercular subjects

They were divided into two groups. (A) pulmonary and (B) extra pulmonary. The tubercular (TB)

subjects (irrespective of age, sex and socio-economic status) attending the Out Patient Department of B.S. Medical College And Hospital, Bankura (W.B), India; and also TB subjects admitted in the Isolation Ward of the same hospital were taken into account. Multi drug resistant (MDR) TB cases were not considered in this study. In these TB subjects, the anti TB drug (A-TB) therapy was started within 0-15 days.

N.B (*Nota bene*)

In this study, all the subjects (groups-1, 2 and 3) having normal liver and kidney function, normal serum glucose level (that is not suffering from diabetes mellitus) were considered.

Collection of blood

About 2 mL of blood was collected from each subject by venipuncture and the blood was left to clot; the serum so obtained was stored in the refrigerator at 2-4°C until assayed. It is to mention that the assay for GS in serum was done on the same day after preparation of the serum. Serum was also collected in the same procedure after exactly thirty (30) days in tubercular subjects with usual treatment (A-TB drugs). The tubercular subjects were under Directly Observed Treatment (DOT) programme under Revised National Tuberculosis Control Programme (RNTCP).

Methods

Assay of serum GS

The highly specific method of transfer reaction was used for measuring GS activity[12]. The γ–glutamyl hydroxamate so formed by the reaction; L-glutamine+Hydroxylamine $\longrightarrow \gamma-$glutamyl hydroxamate+NH_3; in presence of sodium arsenate and ADP; is made to react with ferric chloride ($Fecl_3$) to form orange brown coloured γ-glutamyl hydoxamate-Fe^{+3} complex. The OD of the product was measured at 540 nm. The GS activity in milli Units (mu)/mL was determined from a standard curve drawn with a pure sample of γ-glutamyl-hydroxamate. One unit of enzyme is equivalent to the amount of glutamine synthetase that can produce one micro-mole of γ-glutamyl-hydroxamate per minute at 37°C.

The capacity of L-methionine-S, R sulfoximine (MSO) to inhibit GS activity was determined. MSO is a well defined GS inhibitor having greater specificity for *M. tuberculosis* GS than for mammalian GS[8]. MSO is very much soluble in water or transfer reaction.

Initially, the serum collected at first time from tubercular subject was added to the transfer reaction in definite proportion which did not contain L-glutamine, the substrate, (hence it might be called modified transfer reaction ingredients) and then to this was added MSO at a concentration of 5, 50 and 500 μM, respectively. The mixture was incubated at 37°C for two hours. The incubated mixture was then added separately to a standard transfer reaction mixture and incubated at 37°C. It was then subjected for the usual procedure for measuring the concentration of γ-glutamyl hydroxamate. The result so obtained was expressed as a percentage of the initial serum GS activity in tubercular subjects that is without any incubation of serum with MSO. This part of the study was exclusively meant only to determine the percentage of activity reduction of GS in serum of tubercular subjects when incubated with different concentrations of MSO. The level of serum GS as obtained on first time for the tubercular subject was taken as 100% for the individual. Then after the incubation of the said serum with different concentrations of MSO (5, 50 and 500 μM, respectively) the serum GS was assayed. The activity of serum GS so obtained after incubating serum with MSO was expressed individually as a percentage of initial level taking the initial level (that is without any incubation with MSO) of serum GS activity as 100%. For example, let the initial level of serum GS of a tubercular subject be 10 mu/mL and the serum GS activity after the said incubation with MSO for the same individual be 2 mu/mL. Then, taking the initial value (that is 10 mu/mL) as 100%, the percentage activity of serum GS becomes 20% after incubation. The percentage activity so obtained individually was computed collectively for tubercular subjects and expressed as Mean value ± Standard deviation.

In the similar fashion, purified human liver GS was also assayed after incubating it with MSO of similar concentration as done with serum. The result was expressed as percentage of initial value of purified human liver GS, taking the supplied standard purified human liver GS concentration value as 100%.

Results

All the patients with active tuberculosis, whether pulmonary or extra pulmonary and A-TB drugs started between 0-15 days; had detectable GS activity in their sera; whereas none of the normal control subjects or lung disease control subjects (not suffering from TB) had detectable GS in their sera (Tables 1 & 2). In all the patients with tuberculosis, both pulmonary and extra-pulmonary, the serum GS activity declined

significantly with anti TB drugs, as reflected by the lower values of GS activity in the second sample of patient's 'sera obtained after additional thirty days' treatment with anti TB drugs. The serum GS activity reduced to a considerable extent when the serum of tubercular subjects was incubated with MSO. At a dose of 5, 50, 500 µM of MSO, the serum GS activities were reduced to 20.2±2.4%, 14.4±1.3% and 4.3±0.7%, respectively, of the initial activity (the initial activity was taken as 100%) (Table 3). Therefore, while increasing the dose of MSO the degree of mycobacterial GS activity reduction was increased. However, the purified human liver GS, when incubated with the similar doses of MSO as control, showed activity of 84%, 73% and 57%, respectively, (the initial activity was taken to be 100%) (Table 3).

It is calculated that with further treatment (with A-TB drugs) for another one month, there is a fall of

Table 1 — Serum GS activity in normal control subjects (Group-1)

No. of subjects	Age range of the subjects (years)	Level of GS in the serum (mu/mL)
23	12 – 48	Not detectable at all in all the subjects
Serum GS activity in lung disease control subjects (Group-2)		
26	14 – 52	Not detectable at all in all the subjects

Table 2 — Serum GS activity in pulmonary tubercular subjects (Group 3A)

Sr. No.	Age (years)	Sex	Sputum positivity for TB bacilli	Serum GS level in mu/mL	
				At 0-15 days of treatment with A-TB drugs	Additional treatment for 1 month with A-TB drugs
1	36	F	No	7.67	2.13
2	45	F	Yes	10.60	3.14
3	24	M	Yes	16.87	2.67
4	42	M	Yes	17.23	2.73
5	32	M	No	6.82	2.58
6	18	M	Yes	12.84	3.69
7	28	F	Yes	15.68	3.60
8	47	M	Yes	17.12	2.94
9	28	F	Yes	15.62	2.84
10	37	F	Yes	14.84	3.12
11	35	M	Yes	11.46	2.86
12	30	M	Yes	17.80	2.47
13	41	M	Yes	12.40	3.08
14	35	M	Yes	11.46	2.63
15	33	F	Yes	16.85	3.14

Serum GS activity in extra pulmonary tubercular subjects (Group 3B)

Sr. No.	Age (yrs)	Sex	Diagnosis	Serum GS level in mu/mL	
				At 0-15 days of treatment with A-TB drugs	Additional treatment for 1 month with A-TB drugs
1	40	F	Both sided tubercular pleural effusion	10.04	2.27
2	18	M	TB lymphadenitis	7.96	2.94
3	49	M	Spinal TB	15.35	4.07
4	9	F	TB lymph gland	16.32	5.82
5	53	M	Intestinal TB	11.46	2.84
6	62	M	Rt sided tubercular pleural effusion	14.32	3.14
7	14	F	TB lymph gland	13.83	2.72
8	42	M	TB hip jt.	16.48	3.86
9	8	F	TB meningitis	14.87	3.37
10	54	M	Lupus vulgaris	10.22	2.78
11	11	M	Miliary TB	13.37	4.80
12	37	F	Rt sided TB pleural effusion	6.72	2.08
13	34	M	Urinary bladder TB	11.80	3.14
14	22	F	Rt sided TB pleural effusion	12.90	4.83
15	10	M	TB meningitis	15.75	4.92

Table 3 — Percentage of GS activity when incubated with MSO

Concentration of MSO	GS activity (percentage of initial level)	
	Serum of pulmonary and extra pulmonary tubercular subjects (treatment started between 0-15 days) Total no. of subjects pulmonary TB-15 extra pulmonary TB-15	Purified human liver GS
NIL	100%	100%
5 µM	20.2±2.4%	84%
50 µM	14.4±1.3%	73%
500 µM	4.3±0.7%	57%

serum GS activity by an average of 74.1±3.8% in patients with pulmonary TB and 72.8±2.8% in patients with extra-pulmonary tubercular subjects. It is also noteworthy that almost similar observation had been noticed in both pulmonary and extra-pulmonary tubercular subjects.

Discussion

As reported earlier, the decrease in serum GS activity in tubercular subjects was exaggerated more significantly with oral zinc supplementation (50 milligrams of elemental zinc daily) for one month along with A-TB drugs[13].Oral supplementation of zinc along with A-TB drugs in tubercular subject destabilizes the decompartmentalisation of iron and hastens the process of compartmentalisation of iron. Thus, it helps to prevent *M. tuberculosis* from getting iron[14], which is very much needed as cofactor for the iron-cofactored superoxide dismutase (SOD) secreted extra-cellularly by the pathogenic mycobacteria to get the soluble oxygen by dismutation reaction which is very much needed for this obligate aerobe[15].

As also reported earlier, glutamine synthetase is an essential enzyme for the survival and growth of *M. tuberculosis*[5]. The GS enzyme which lacks a leader sequence and is located in the bacterial cytoplasm, is released by the organisms growing in the medium(broth) as well as while growing within phagosomes in human mononuclear phagocytes. The abundant release of GS can be correlated with the prevalence of considerable amount of poly-L-glutamine/glutamate in the cell wall of these organisms[16].

In an earlier report, the average concentration of GS in normal control serum sample (n=35) was 36±27 pg/mL and in the serum of healthy blood donors (n=24), it was 50±19 pg/mL. In that study, sandwich enzyme immunoassay and immunoblotting were applied to assay GS in serum. In the same study, the serum GS level was reported in patients with amyotrophic lateral sclerosis (n=8) as 116±6.2 pg/mL; with Alzheimer's disease (n=9) as 111±53 pg/mL; with vascular dementia (n=15) as 72±59 pg/mL; with Parkinson disease (n=5) as 77±32 pg/mL and with schizophrenia (n=4) as 74±32 pg/mL.

These values are very low when compared with the serum GS level in tubercular subjects and even undetectable by transfer reaction, the assay procedure applied in the present study. Therefore, the very little increase in the endogenous GS in serum with the said ailments will not interfere at all with this diagnostic for tuberculosis. So, the detection of serum GS by transfer reaction might be a useful parameter in the early and positive diagnosis of tuberculosis[17].

The specificity in the assaying procedure for GS activity was cent percent. The normal control subjects as well as lung disease control subjects had undetectable level of serum GS activity. However, the accuracy for assaying the mycobacterial GS activity can be increased by incubating the subject serum with MSO, a selective inhibitor of mycobacterial GS rather than human (mammalian) GS activity.

So, the assay for serum level of GS will provide a faithful diagnosis for active tuberculosis. A gradual decrease in the serum level of mycobacterial GS (during the course of treatment with A-TB drugs) will indicate drug sensitivity. The assay is also helpful for detecting the reactivation of tuberculosis in latently infected subjects. Failure of serum GS level to come down from the very beginning would indicate primary drug resistance whereas a rebound in serum GS level after the fall primarily would indicate secondary drug resistance.

In the present scenario, to have a substantial impact on the TB pandemic, a simple and rapid method for mycobacterial GS assay in the serum of suspected subjects might be instituted for field health workers. This will facilitate the early diagnosis and differentiation of the patients with TB from other patients having similar symptoms but suffering from other lung ailments. This would reduce the transmission of the disease substantially.

The rapid serological test for the qualitative and quantitative analysis for serum GS might be done by using ELISA or latex agglutination. The wells might be coated with monoclonal or polyclonal antibodies specific to *M. tuberculosis* GS. Now, a measured amount of serum (with or without mycobacterial GS)

might be added to the well and incubated at 37°C. To this, might be added a mixture containing glutamine, sodium arsenate, manganese chloride, hydroxylamine hydrochloride (neutralized with NaOH), ADP, tris hydrochloric acid in definite proportion and concentration. Now, after the proper incubation, the reaction might be stopped by the addition of definite volume of mixed reagent containing equal volumes of 24% trichloroacetic acid, 6(N) HCl and 10% $FeCl_3$ in 0.02(N) HCl. If GS is present in the serum, an orange brown coloured γ-glutamyl hydroxamate-Fe^{+3} complex is formed, whose absorbance is measured at 540 nm spectrophotometrically. A standard curve is to be prepared with a pure sample of γ-glutamyl hydroxamate to determine the serum GS level in test serum in mu/mL. The specificity of the test might be increased with the use of MSO, a selective inhibitor of mycobacterial GS. By this technique, not only the definite diagnosis of active tuberculosis (both pulmonary and extra pulmonary) might be done; but also diagnosis of latent tubercular cases might be made. It also might enable us to know the success of the course of anti-tubercular drugs, or the development of primary or secondary drug resistant cases as mentioned earlier.

For rapid field study to know the prevalence rate of tuberculosis at a particular region, a card impregnated with GS active substrate and reagent when kept in contact with a drop of test serum; the presence of *M. tuberculosis* GS would be evidenced by a visible colour change (orange brown) of the substrate.

Therefore, with this assay there will be quick identification and rapid treatment of TB patients, which will result in the reduction of transmission of TB below the threshold needed to maintain the pandemic. Thus, the transmission of TB would eventually cease in theory.

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