



METABOLIC CHANGES IN *MYCOBACTERIUM TUBERCULOSIS* DURING NUTRIENT STARVATION CONDITIONS

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ABSTRACT

Latent *Mycobacterium tuberculosis* exhibits metabolic pathways different from normal bacilli. Mycobacteria undergo metabolic shift leading to alteration in expression of vital cell proteins like catalase, urease etc., during nutrient starvation conditions. In this study, we determined the activity of catalase and urease in nutrient starved cultures. It was observed that catalase activity declines to 87% and 91%, when compared to log phase cultures as determined by bubble height and spectrophotometric methods, respectively. On the contrary, urease activity increases 18 fold in 42 h nutrient starved mycobacteria as compared log phase bacilli. Latency in nutrient starved bacilli was confirmed by respiration test and sensitivity tests. We also studied the susceptibility of latent bacilli to RNI under nutrient starvation conditions. Bacilli become resistant to acidified sodium nitrite induced RNI (at 0.05, 0.5, 1 µg/ml concentrations) in latency induced by nutrient starvation. Latency in nutrient starved bacilli was confirmed by respiration test and sensitivity tests. These findings suggest that changes in levels of catalase, urease and RNI susceptibility may play critical role in latent TB.

KEY WORDS: *M. tuberculosis*, catalase, urease, latent TB, nutrient, starvation



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INTRODUCTION

World Health Organization (WHO) database estimates that about one-third of world population is infected with latent tuberculosis infection (LTBI) and approximately 8 million cases of active tuberculosis (TB) are pulled out from these infected individuals annually¹. These infected individuals serve as huge pool for reactivation to active TB cases. TB is one of the leading causes of death amongst all the infectious diseases. Latent tuberculosis (LTB) is an asymptomatic infection where bacilli remain in a metabolically quiescent state and persist in the host for long periods of time. The latent bacilli may reactivate to active disease when host immunity is compromised². Host immune response arrests the growth in lungs by forming granuloma. Inside the granuloma, mycobacteria can survive for years by switching to dormant state. Dormant mycobacteria alter the gene expression pattern when compared to log phase bacilli. This altered gene expression pattern leads to changes in metabolic pathways helping the mycobacteria to persist in non-replicating state within caseous necrotic center³. As a result, many drugs like rifampicin are not active against non-replicating bacilli; however, some drugs like clofazimine has shown activity in vivo and in vitro against non-replicating *Mycobacterium tuberculosis*⁴⁻⁵.

In non-replicating state, various metabolic changes take place in mycobacterial cells due to shift in protein expression profiles including enzymes⁶. The alterations may be upregulation or downregulation of enzymes, based on their role in during latency. Various enzymes like catalase and urease are expressed in *Mycobacterium tuberculosis* (*M. tuberculosis*) under normal growth conditions; however, changes in their expressions under adverse conditions such as nutrient starvation have not been elucidated. Catalase, an enzyme responsible for negating an action of oxidative free radicals, protects the bacilli from noxious oxygen by-products like superoxide

anion (O_2^-) and hydrogen peroxide ($H_2O_2^-$) generated during microbial respiration due to incomplete reduction of oxygen⁷. Urease catalyzes the breakdown of urea to carbon dioxide and ammonia. The released ammonia alters the phagosomal pH to elude the fusion of lysosome and phagosome. Urease also, plays an important role in virulence of *M. tuberculosis* like other microorganisms⁸. During nutrient starvation induced latency, the changes in catalase and urease activity of *M. tuberculosis* H37Rv have not been fully revealed. Under stationary phase, *M. tuberculosis* expresses specific protein alpha crystallin. The 16 KD alpha crystallin protein plays the most significant role in induction of nonreplicating persistence (NRP) state in infected humans, and is expressed at the beginning time of NRP-1 and is maintained in culture during hypoxic conditions⁹⁻¹¹.

Macrophages protect the host from pathogens by releasing reactive nitrogen intermediate (RNI) which is one of the vital host defense mechanisms. Therefore, RNI plays indubitably a crucial role in host defense system in active TB. Moreover, RNI produced by macrophages and other cells, has been shown to be effective against actively growing bacilli¹². It is also one of the factors in granuloma that compel bacilli to shift into latency. Studies in murine macrophages has illustrated the role of RNI in curbing the mycobacterial infection to the logical extent, nevertheless, it still remains the subject of debate¹³. In latent state, bacilli adopt to adverse conditions by changing and/or shutting down various metabolic pathways¹⁴. However, action of RNI against latent *M. tuberculosis* under nutrient starvation conditions is still unknown.

MATERIALS AND METHODS

Test Strain: *M. tuberculosis* H37Rv strain (Tuberculosis Research Centre, Chennai, India) was grown in Middlebrook 7H9 liquid

medium (7H9; HiMedia, India) supplemented with 10% albumin, dextrose, and catalase (ADC; HiMedia, India) to mid-log phase and frozen in aliquots at -70°C until needed in further *in vitro* assay.

Nutrient starvation conditions: *M. tuberculosis* H37Rv culture was grown to mid log phase in Middlebrook 7H9 media. The culture was pelleted by centrifugation, washed three times with phosphate buffer saline (PBS) and finally suspended in PBS. These culture suspensions were incubated for different time intervals at 37°C ¹⁵.

Catalase activity determination: Two methods - bubble height and spectrophotometric methods were employed for determination of catalase activity in nutrient starved *M. tuberculosis* ¹⁶. In bubble height method, 5 ml of mixture of 15% H_2O_2 and 5% Tween 80 was added to pellets of cells under nutrient starvation for 0.5, 1, 2, 3, 18, 24, 42 h in specific tubes in triplicate. Then, maximum height of bubbles on the surface of solution was measured in the next 10 minutes. In spectrophotometric method, *M. tuberculosis* culture was kept under nutrient starvation conditions for different time intervals i.e. 0, 0.5, 1, 2, 3, 18, 24, 42 h and then these nutrient starved cells were lysed by using MagnaLyzer (Roche applied science product). After lysis, 100 μl aliquots of the bacterial cell lysates were added to 3 μl H_2O_2 solutions (30%) and volume was adjusted to 1 ml with 50 mM potassium phosphate buffer. All the determinations were performed in triplicate. The absorbance of solutions was measured at 240 nm.

Urease activity: Urease media was prepared as per the standard protocol of the laboratory (yeast extract-0.1 g, Monopotassium Phosphate- 9.1 g, Dipotassium Phosphate- 9.5 g, urea- 20 g, Phenol Red- 0.01 g in 1 liter of purified water). Nutrient starved mycobacterial cells were lysed by using MagnaLyzer after exposure to nutrient

starvation conditions for 0, 0.5, 1, 2, 3, 18, 24, 42 h time intervals. Then, 1 ml each of crude cell lysates was added to 5 ml of sterile urease broth for different intervals and absorbance was taken after 30 minutes at 557 nm ¹⁷.

Nutrient starvation induced latency confirmation: The latency in nutrient starved *M. tuberculosis* was confirmed by three tests viz. Respiration test and Sensitivity test (sensitivity to isoniazid (INH) and clotrimazole) and expression of 16 KD alpha crystallin protein.

a. Respiration test: Respiration of *M. tuberculosis* during nutrient starvation conditions was studied by employing methylene blue test. In this test, methylene blue was added to 10 ml each of log phase culture, starved cell culture and PBS to give final concentrations of 1.5 g/ml in each of the tubes. The tubes were tightly closed and incubated at 37°C . The change in color was observed and interpreted accordingly ¹⁵⁻¹⁸.

b. Sensitivity test: In 96 well plates, 1 ml of nutrient starved latent mycobacteria were added to wells to determine the sensitivity of INH and clotrimazole. Sterile 0.1 ml of INH solution was added to wells containing nutrient starved *M. tuberculosis* to give final concentration of 10 $\mu\text{g}/\text{ml}$ and 5 $\mu\text{g}/\text{ml}$ of INH in triplicates ¹⁸. In the same way, clotrimazole was added to achieve final concentration of 0.6 $\mu\text{g}/\text{ml}$ and 1.2 $\mu\text{g}/\text{ml}$ of clotrimazole in triplicate. Vehicle control was also incorporated, which is devoid of any drug. Then, the plate was incubated for 7 days at 37°C . After 7 days of incubation with drug, the growth of mycobacterial culture was determined by BACTEC 460 TB system (Becton Dickinson, USA). It determines growth index (GI) of *M. tuberculosis* which is converted to percent growth inhibition ¹⁸.

c. Expression of 16 KD alpha crystallin protein: The expression of specific protein alpha crystallin (16 KD) in nutrient starved *M. tuberculosis* bacilli was carried by SDS-PAGE

Electrophoresis. The procedure of SDS-PAGE Electrophoresis was established and validated in the laboratory.

Cell disruption & protein extraction: The nutrient starved cultures were heat-killed at 80° C for one hour, consequently the bacterial pellet resulting from centrifugation was washed three times with sterile phosphate buffered saline PBS -T (comprise 0.2 % Tween-80) and then twice with sterile PBS. Pellet was re-suspended in cell disruption Tris-HCL (pH=8.8) buffer at an approximate concentration of about 30 % (w/v). An equal volume of 110-micrometer glass beads was added, and sample was vortexed for about 5 min at high speed. Sample was centrifuged at 20000 g (at- 4° C) and the superficial layer carefully transferred to Eppendorf tube. The cell-free extracts prepared under these conditions contained about 1 mg/ml of total proteins as determined by the Bio-Rad protein assay Kit.

SDS-PAGE Electrophoresis: Protein was mixed with equal volume of loading Tris buffer comprise (pH=6.8, 0/064 M containing 2% SDS, 10 % glycerol, 5% mercaptoethanol , and 0/0025 % bromophenol blue), were denatured in a boiling water bath and the denatured samples (30 µl/ well) were fractionated by SDS PAGE. The total protein was stained with 0.25 % Coomassive brilliant blue, and the results were compared with

parallel molecular weight markers that had been purchased from Bio Rad.

Acidified sodium nitrite (ASN) assay: Acidified sodium nitrite (ASN) assay was standardized in laboratory conditions. 1 ml each of log phase cultures of *M. tuberculosis* H37Rv was taken in micro wells. Then, 0.2 ml of acidified sodium nitrite solution (pH 5.3) were added into log phase cultures of *M. tuberculosis* H37Rv to achieve final concentration of 0.05, 0.5, 1 µg/ml. These solutions were incubated at 37°C for 24 h. After incubation, cultures were inoculated into BACTEC vials and Growth Index (GI) was determined.

Susceptibility of latent *M. tuberculosis* H37Rv to reactive nitrogen intermediate (RNI) was determined by acidified sodium nitrite (ASN) assay¹⁴. In this method, *M. tuberculosis* was subjected to nutrient starvation as described previously for 42 days to induce latency. Freshly prepared acidified sodium nitrite solution (0.2 ml, pH 5.3) was added to micro wells containing 1 ml of starved bacilli to achieve a final concentrations of 0.05, 0.5, 1 µg/ml. Control wells contained bacilli and vehicle (water, pH 5.3) without sodium nitrite¹². Then, these solutions were incubated at 37°C for 24 h. After incubation, the treated and control cultures were inoculated into BACTEC vials and Growth Index (GI) was determined.

RESULTS AND DISCUSSION

Estimation of catalase activity: In bubble height method, height of bubbles on the surface of solutions with time of starvation has been shown in fig. 1. In this experiment, it was observed that height of bubbles dropped in starved bacilli with increase in time of

starvation as compared to that of normally growing bacilli. In the first half hour of starvation, there was sharp decline (approximately 40%) in height of bubbles in starved bacilli whereas height of bubbles gradually decreased to 16.6% over next 18 h and after that it remained almost constant.

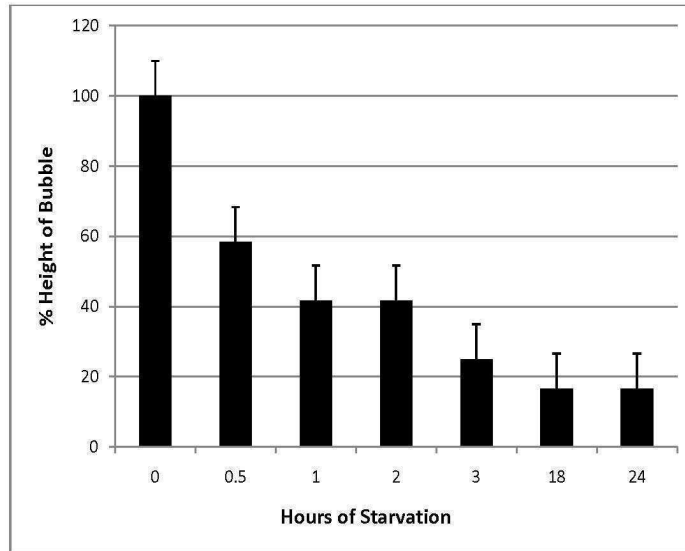


Fig. 1: Bubble height method: % height of bubbles has been shown against the starvation time. % height of bubbles \pm SEM has been plotted

In spectrophotometric method, average absorbance has been plotted against starvation time (fig. 2). In this study, initially the absorbance of solution was very low but it increased gradually with time. The rise in absorbance was slow till 1 h, however, after that absorbance increased sharply in crude lysates as shown in the fig. 2.



Fig. 2: Spectrophotometric assay: Absorbance has shown against the starvation time. Absorbance \pm SEM has been plotted

Estimation of urease activity: In urease estimation of starved mycobacteria, there was rise in activity of urease enzyme in crude lysate of starved mycobacterial cells. As shown in the fig. 3, the urease activity increases gradually over 3 hr of starvation, however, there was sharp rise in urease activity afterwards. In next 42 h of starvation, there was about 18 fold increase in urease activity in crude lysate of mycobacterial cells.

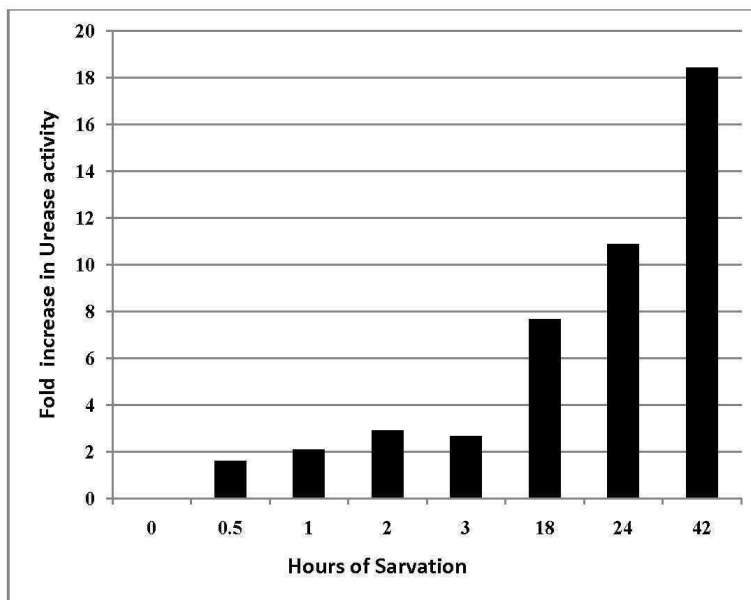


Fig. 3: Urease activity: Absorbance has shown against the starvation time. Absorbance \pm SEM has been plotted

Nutrient starvation induced latency confirmation

a. Respiration test: As shown in fig. 4, the tube containing starved mycobacterial cells did not decolorize and it remained as blue as in negative control (PBS), whereas, the tube containing log phase culture decolorized within 17 days of incubation¹⁵.

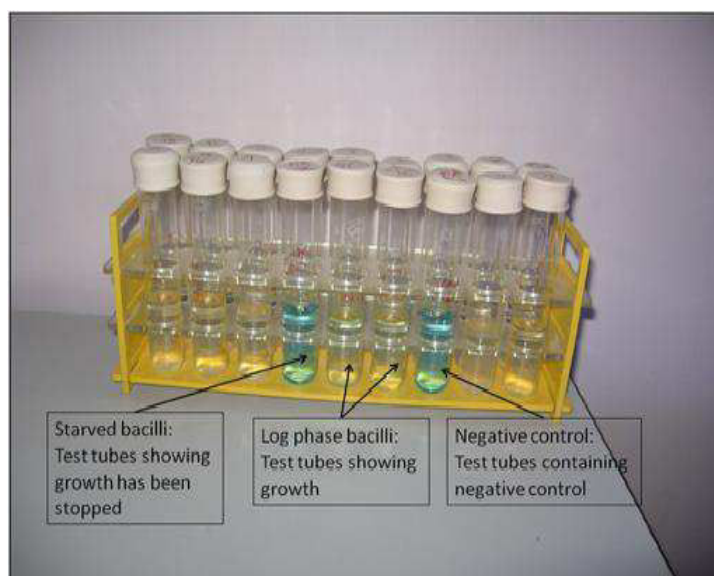


Fig. 4: Respiration test: Starved bacilli showing the growth of bacilli has been stopped as compared to the log phase bacilli

b. Sensitivity test: In this confirmatory assay, % growth of INH treated mycobacteria under nutrient starvation (at concentrations of 5 μ g/ml and 10 μ g/ml) has no statistically significant growth as compared to % growth of mycobacteria treated with vehicle control (fig. 5). In addition,

there was no growth (i.e. 0% growth) in clotrimazole treated bacilli at 0.6 $\mu\text{g/ml}$ and 1.2 $\mu\text{g/ml}$ concentrations.

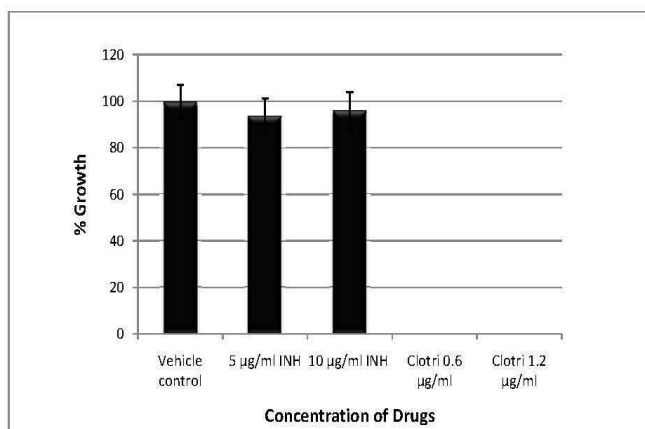


Fig. 5: Effect of INH and Clotrimazole on latent bacilli.
 % Growth \pm SEM has been plotted with various conc. of INH and Clotrimazole.

c. Expression of 16 KD alpha crystallin protein: The nutrient starved mycobacteria were examined for expression of alpha crystallin in SDS-PAGE compared to log phase culture. As shown in figure 6, expression of alpha crystallin (16 KD) protein was not detected in nutrient rich culture, but 16 KD alpha crystallin protein band was detected in nutrient starved culture.

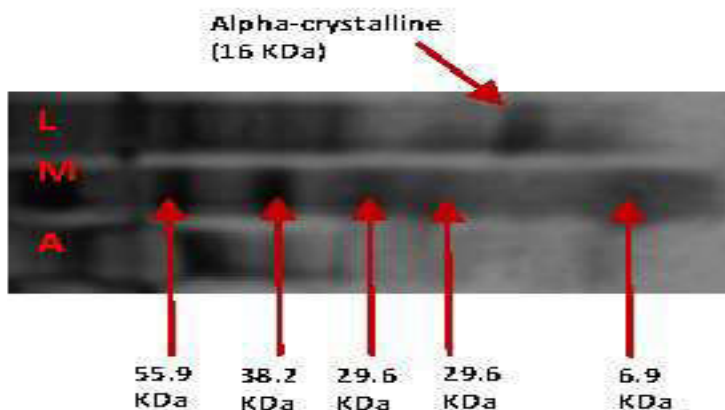


Fig. 6: Expression of alpha crystallin in gel electrophoresis

d. Determination of RNI activity on latent bacilli under nutrient starvation conditions

In standardization of RNI susceptibility test, the killing of log phase culture by RNI induced ASN (at concentrations of 0.05, 0.5, 1 $\mu\text{g/ml}$) is significant ($p < 0.05$) as shown in figure 7.

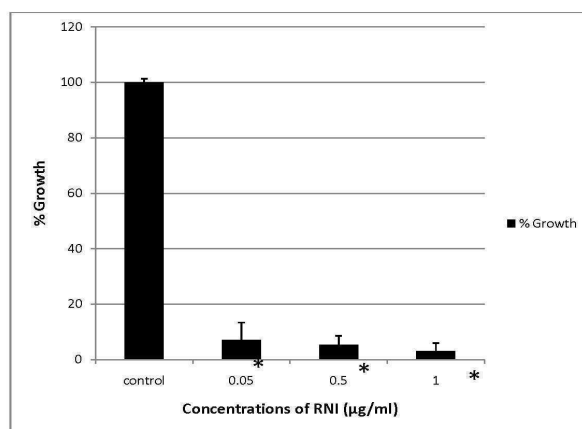


Fig. 7: Effect of RNI on log phase bacilli: % Growth of bacilli compare to the control has been shown. % Growth ± SEM has been plotted with conc. of RNI

In brief, Growth Index (GI) values of the sodium nitrite induced RNI and vehicle control (without RNI) treated latent bacilli were determined. Percent growth and concentration of RNI has been plotted as shown in fig. 8.

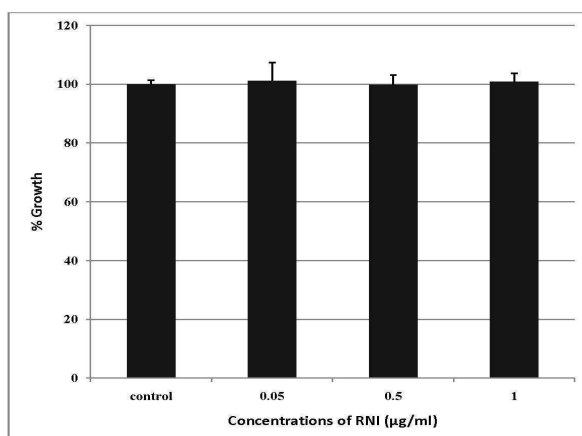


Fig.8: Effect of RNI on latent bacilli: % Growth of bacilli compare to the control has been shown. % Growth ± SEM has been plotted with conc. of RNI

Estimation of catalase activity

Drop in height of bubbles in starved bacilli is attributed to the decrease in activity of catalase enzyme present in the microbial cells that breaks down H_2O_2 to release oxygen as bubbles in the solution. The height of the deposited bubbles is directly proportional to the catalase activity in bacilli. Moreover, it also suggests that there was drastic decline in catalase activity in the first half hour of starvation, whereas catalase activity slowly goes down after half an hour of starvation. Archuleta et al. also showed the similar

observations in *M. avium* during starvation conditions¹⁴.

In spectrophotometric method, the rise in absorbance is attributed to less breakdown of H_2O_2 in the solution as a result of low catalytic activity of cell lysates. The absorbance is dependent upon concentration of H_2O_2 which in turn is inversely proportional to catalase activity *i.e.* lower the absorbance of H_2O_2 higher the catalase activity. During metabolism, reactive oxygen intermediates (ROI) are generated in bacilli, which are detrimental to cells as they damage membrane lipids,

proteins, and DNA of cells. Catalase protects the cells from these oxidative stress induced by host immune cells. Due to minimal mycobacterial respiration during nutrient starvation; free oxygen radicals generation is minimized that can be elucidated by decreased catalase activity. The limitation of both spectrophotometric as well as bubble height methods is that the role of specific genes in the metabolic shift cannot be explained by these methods; as both the methods determine total catalase activity in whole cells.

Estimation of urease activity

Increase in urease activity may be a survival strategy for mycobacteria in stress conditions like starvation. Urease present in crude cell lysates hydrolyzes urea resulting in decrease in absorbance of the solution. This can be ascribed to increased activity of urease in crude cell lysates of starved bacilli during nutrient starvation conditions. There was increase in urease activity up to 18 fold in starved cultures as compared to log phase cultures, however; after 42 h, it remained constant. Urease attributes the survival of mycobacteria by evasion of phagolysosome maturation inside macrophages⁶. Since urease assist bacilli to persist in macrophages, mycobacteria upregulate the urease activity under stern conditions like nutrient starvation. Though, urease assists bacilli in persistence inside macrophage; exact role of urease in nutrient starvation has not been fully understood.

Nutrient starvation induced latency confirmation:

a. Respiration test: These observations imply that oxygen present in the media is consumed by actively growing bacilli (log phase culture), because all the respiration pathways of mycobacteria consume the oxygen. But bacilli do not consume oxygen under starvation conditions as they shut down the respiration pathways by reducing the

expression of enzymes involved in metabolism. This supports global metabolic switch of bacilli to inactive state⁶.

b. Sensitivity test: Mycobacterial culture under nutrient starvation conditions was found to be resistant to INH (at 5 µg/ml and 10 µg/ml), which endorses the results of *Betts et al*¹⁵. Moreover, the nutrient starved *M. tuberculosis* culture is sensitive to clotrimazole (0.6 µg/ml and 1.2 µg/ml)¹⁵⁻¹⁸. The change in sensitivity towards the various drugs (like INH, clotrimazole) is characteristic of latent mycobacteria; therefore, the change in sensitivity is an indicator of switch of bacilli into latency.

c. Expression of 16 KD alpha crystallin protein: The protein, alpha crystalline, is predominantly expressed by MTB during stationary growth or subjected to oxygen deprivation in NRP stages, and can account for up to 25% of total bacillary proteins expressions in these circumstances¹⁹⁻²⁰. The induction of alpha crystallin synthesis in bacilli during nutrient starvation suggests a critical role of this protein in persistence of bacilli without replicating in the hostile regions of the host tissues. The 16-kDa protein might be an important antigenic target during bacillary latency²⁰.

d. Determination of RNI activity on latent bacilli under nutrient starvation conditions

It implies that latent mycobacteria develop resistance to RNI to stay alive in stress induced by nutrient starvation. Therefore, macrophages cannot eradicate latent bacilli present in the granulomatous foci by forming RNI. However, it has been widely reported that RNI play critical role in controlling the reactivation of latent mycobacteria to active TB¹³. Therefore, host immune cells cannot eliminate bacilli from host body through RNI approach. Further, the role of RNI induced by macrophages in latent mycobacteria under nutrient starvation conditions need to be investigated.

CONCLUSIONS

Catalase activity decreases in *M. tuberculosis* H37Rv under nutrient starvation conditions which is evident from the catalase determination by both the methods: bubble height and spectrophotometric methods. When bacilli undergo nutrient starvation there is a sharp decline in free oxygen radical generation that is determined in terms of decreased catalase activity. Therefore, catalase activity plummets as a part of global metabolic shift of mycobacteria. On the other hand urease activity increases in *M. tuberculosis* H37Rv under nutrient starvation conditions. Hence,

urease may assist persistence of bacilli under nutrient starvation conditions. This implies that mycobacteria shut down respiratory pathways under nutrient deficient conditions and endorse the global metabolic shift. During nutrient starvation *M. tuberculosis* increases the alpha crystallin protein expression. *M. tuberculosis* also becomes resistant to RNI to escape stress conditions like nutrient starvation. Therefore, latent bacilli may not be eradicated from the host by RNI only. More investigations are required to correlate the levels of catalase, urease, alpha crystallin and RNI susceptibility in *M. tuberculosis* during nutrient starvation conditions and their potential role in latent TB

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