Molecular Characterization and Bioinformatics approach of Tuberculosis Infection prevalent in Methyl Isocyanate affected population in Bhopal

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Summary

The study group consisted of 50 patients suspected to be positive for MTB were chosen for the study. None of them were positive for *Mycobacterium tuberculosis* complex treated through culture and smear and divided into different groups viz. MIC affected and unaffected patients, pulmonary and extra pulmonary and age wise. Real time assay performed using fluorescence resonance energy transfer hybridization (FRET) probes showed that mean bacterial load of MIC affected patients was more than Non-MIC patients in all groups . Results clearly indicate that the patients affected by MIC are more prone to get infection from tuberculosis. The result so varies, because of suppressed immunity in MIC affected population.

From the analysis of MIC in CTD database, observed that methyl isocyanate causes tuberculosis and the proteins responsible for tuberculosis are primarily IL4 and IFNG found in many pathways.

A combinatorial diagnostic approach for rapid detection and characterization of MTB might provide specific therapeutic strategies for prevention and treatment of the infection in future.

Key words: MIC, MTB, FRET

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Introduction

Bhopal gas tragedy was an industrial disaster that took place at the Union Carbide Pesticide Plant in Bhopal, M.P, at midnight on 2-3 December 1984.The plant had accidently released Methyl Isocyanate gas. Methyl Isocyanate (MIC) is a highly toxic, hazardous and irritating organic compound with the molecular formula C_2H_3NO. The inhalation or ingestion of MIC can cause acute effects as pulmonary edema, bronchitis, bronchial pneumonia, respiratory tract irritation, difficulty in breathing, blindness, nausea, gastritis, liver and kidney damage **[1]**. Its chronic effects are disorders of reproductive system including leukorrhea, pelvic inflammatory disease, excessive menstrual bleeding, suppression of lactation, stillbirths and spontaneous abortions **[2, 3]**.

People exposed to high levels of MIC are more prone to get infected by *Mycobacterium tuberculosis* (MTB). MTB is highly contagious disease, spread via aerosols. *Mycobacterium tuberculosis* complex (MTBC) is a group of ubiquitous intracellular pathogens responsible for 2-3 million deaths per year. India reported a significant high morbidity and mortality due to tuberculosis (TB) contributing one-fifth of the global cases with 325 172 deaths reported in the 2005 cohort **[4, 5]**.

A major development in the diagnosis of TB was the introduction of several nucleic-acid amplification (NAA) techniques, such as the polymerase chain reaction (PCR) that has been widely evaluated. The PCR can provide useful fast diagnostic test equivalent to bacteriological cultures **[6].**

Several authors have developed real-time PCR assays that provide rapid detection of target sequences of *Mycobacterium tuberculosis* in clinical specimens with variable sensitivity and very high specificity **[7, 8, 9, 10]**.

The proposed objective of the present study is to revaluate and compare the evidence of tuberculosis infection in Cohort exposed to MIC in Bhopal on different group by using PCR technology and bioinformatics approach.

Materials and Method

Reagents: For isolation of MTBC DNA fluid ,DNeasy Blood & Tissue kit (QIAGEN, Hilden, Germany) was used. Real-time assay was performed in thus extracted DNA with the help of LC PCR MTB detection kit using Roche Light Cycler 2.0 (LC) (Roche Molecular Diagnsotics, Mannheim Germany).

Specimen collection: The study group consisted of 50 patients suspected to be positive for MTB were chosen for the study. None of them were positive for *Mycobacterium tuberculosis* complex treated through culture and smear and which was divided into three groups - (A) MIC affected population (n=35) and Non MIC patients (n=15) (B) Pulmonary MIC patients (n=7) and Non MIC patients (n=2) and Extra pulmonary MIC patients (n=28) and Non MIC patients (n=13) (C) Age wise MIC and Non MIC patients.

For extra pulmonary samples endoscopy biopsy, Formalin fixed tissue samples surgically resected tissues of the gastrointestinal tract were processed for the isolation and identification of the bacterial DNA and for pulmonary samples , pulmonary origin obtained were: pleural fluid and bronchial aspirate and all of these samples were obtained from Bhopal Memorial Hospital & Research Centre and stored at -20°C (processed within 7 days). 10 ml of EDTA mixed blood was collected from LC PCR GITB positive patients .

Extraction MTB DNA from pulmonary fluids : Maximum 7 ml patients sample was added to the falcons tube and 4% NaOH equal the amount to the patient's sample was added and samples were shaked for 15 minutes and then vortexed and centrifuged at 3000 rpm for 20 minutes and the supernatant was discarded.10 ml 1X PBS in each tube was added and vortexed and again centrifuged at 3000 rpm for 20 minutes. The supernatant was discarded without the loss of the pellet and transferred in micro centrifuge tubes (1.5 ml).1 ml 1X PBS was added in each tube and vortexed and centrifuged at 13000 rpm for 10 minutes and supernatant was discarded and 180 µl lysozyme added in the pellet. The samples were incubated at 37º C for 1 hour. Short spin at 2000 rpm for 10 seconds and incubated at 95º C for 15 minutes and again Short spun at 2000 rpm for 10 seconds. 200 µl 100% ethanol was added and pipetted out the mixture in DNaesy mini column tubes and centrifuged at 8000 rpm for 1 minute. The columns were placed in new collection tubes and 500 µl AW1 was added and Centrifuged at 8000 rpm for 1 minute. The columns were placed in new collection tubes and 500 µl AW2 was added. Centrifuged at 13000 rpm for 3 minutes. The columns were placed in new collection tubes and centrifuged at 15000 rpm for 1 minute. The columns were placed in new collection tubes and incubated at 56 º C for 2 minutes. The columns were placed in micro centrifuge tubes and 50 µl AE was added and centrifuged at 8000 rpm for 1 minute.50 µl obtained contained the desired DNA.

Extraction MTB DNA from Extra pulmonary fluids : Formalin fixed and paraffin-embedded (FFPE) specimens were sliced with disposable sterile blades in each paraffin block, de paraffinized twice with 1 ml xylene and ethanol (100%), whereas for the formalin-fixed tissue biopsy and endoscopy samples, the specimens were washed twice in PBS prior to DNA isolation and DNA was extracted from the samples by Proteinase K digestion in combination with DNeasy Blood & Tissue kit following the tissue protocol **[11].**

Quantification of Pulmonary and Extra pulmonary MTB DNA through LC PCR: Real-time assay was performed in thus extracted DNA with the help of FRET probes using LC PCR 2.0 following all necessary instructions. A universal set of primers directed towards most

conserved region of the 16S rRNA gene of the MTBC was used. The sequences of the primers, which recognized a 206-bp region of the 16S rRNA gene, were 5'-ACGGAAAGGTCTCTTCG-3' and 5'-CTTGGTAGGCCGTCAC-3'. The sequence of internal control (IC) oligonucleotideusedwas5'ACGGAAAGGTCTCTTCGGAGATACTCGAGTGGCGAACGGGT GAGTAACACGTGGGTGGGAAGCATGTTTTGTGGTGTAAAGCGCTTTAGCGGTGTGG GATGAGCGTGACGGCCTACCAAG3'.Working master mix was prepared as per the protocol by adding 13.5 µl master mix concentrate, 2 µl Mg 2+ and 0.5 µl (IC). Finally 15 µl of working master mix and 5 µl of the sample were dispensed in each capillary and were run on LC PCR. Absolute quantification of bacterial load was performed by using Light cycler software 4.0 with appropriate quantitative standards (MTBC positive controls) and by following the guidelines for the quantitative analysis on the Light Cycler 2.0 instrument. Quantitative bacterial load means the total amount of bacterial DNA present in the given sample expressed in copies/µl.

Bioinformatics Approach : From the analysis of MIC in CTD database, observed that methyl isocyanate causes tuberculosis and the proteins responsible for tuberculosis are primarily IL4 and IFNG which are found in many pathways. The connecting proteins and the pathways in which are found are described.

Table 1: CONNECTING PROTEINS OF IFNG

PATHWAY	**PATHWAY ID**	**CONNECTING PROTEINS**
Allograft rejection	KEGG:05330	**CD40, CD40L**
Cytokine-cytokine receptor interaction	KEGG:04060	**IFNGR1, IFNGR2**
Jak-STAT signaling pathway	KEGG:04630	
T cell receptor signaling pathway	KEGG:04660	**NFAT, AP1, NFKB**
Chagas disease	KEGG:05142	**IFNGR**
Leishmaniasis	KEGG:05140	**IFNGR1, IFNGR2**
Graft-versus-host disease	KEGG:05332	**FasL**
Malaria	KEGG:05144	**NKC. TCR**

Systemic lupus erythematosus	KEGG:05322	**TCR, CD40, CD40L, BCR, MHC2**
Type I diabetes mellitus	KEGG:04940	**TCR, CD28, FasL, MHC-1, Fas**
Antigen processing and presentation	KEGG:04612	**PA28, TNFα**
Natural killer cell mediated cytotoxicity	KEGG:04650	
Proteasome	KEGG:03050	
Regulation of autophagy	KEGG:04140	**IFNα**
GF-beta signaling pathway	KEGG:04350	**Smad6/7, TNFα**

Table 2 : CONNECTING PROTEINS OF IL4

PATHWAY	**PATHWAY ID**	**CONNECTING PROTEINS**
Allograft rejection	KEGG:05330	CD40L, CD40, TCR, MHC2, BCR
Cytokine-cytokine receptor interaction	KEGG:04060	IL4R, IL2RG, IL13RA1
Jak-STAT signaling pathway	KEGG:04630	
T cell receptor signaling pathway	KEGG:04660	NFAT, AP1, NFKB
Autoimmune thyroid disease	KEGG:05320	CD40L, CD40, TCR, MHC2, BCR
Intestinal immune network for IgA production	KEGG:04672	CD40L, CD40, TCR, MHC, BCR, CD28, ICOS, TAC1, AID,ICOS-L, B7, BCMA, BAFFR
Leishmaniasis	KEGG:05140	iNOS

Asthma	KEGG:05310	CD40L, CD40, TCR, MHC2, BCR FCER1
Fc epsilon RI signaling pathway	KEGG:04664	
Hematopoietic cell lineage	KEGG:04640	

Results and Discussion

Mycobacterium tuberculosis infects one-third of the world's population **[12]** and is the leading cause of death due to any infection worldwide [**13].** Due to the slow growth of *M. tuberculosis*, rapid identification methods using molecular techniques have been developed and utilized in the clinical laboratories.

A positive fluorescence signal in pulmonary and extra pulmonary samples confirming the presence of MTBC DNA. However the utility of real time PCR assay in the known culture and ZN stain positive specimens showed 99% specificity in MTBC diagnosis. The internal control of the real-time PCR assay was positive for all specimens, negating out the existence of possible inhibitions.

The bacterial load of the Methyl isocyanate (MIC) affected patients and Non-MIC patients was compared and on comparison we interpreted that the mean bacterial load of MIC affected patients was more than the mean bacterial load of the Non-MIC patients. Also on further comparison between MIC affected and Non-MIC patients in different groups showed that the mean bacterial load of MIC pulmonary cases was more than Non-MIC pulmonary cases and the MIC extra-pulmonary was more than that of Non-MIC extra-pulmonary cases. Grouping the MIC affected and Non-MIC patients age-wise it was found that MIC affected patients between 0-40 years had a higher mean bacterial load than the Non-MIC patients aged between 0-40 years and the same was observed between patients aged 41-80 years.

Our results (Table 3) in different group wise study; Total MIC and Non MIC patients, Pulmonary and extra pulmonary MIC and Non MIC patients and Age wise MIC and Non MIC patients and Graph clearly demonstrate that the patients affected by MIC are more prone to get infected by tuberculosis. Out of the 50 patients studied 70% were MIC affected and 30% were Non-MIC patients. The result so varies, because of suppressed immunity in MIC affected population. Immune function was studied in exposed subjects at the Indian Toxicology Research Centre (ITRC) in sample after 2.5 months after exposure. No difference in mean immunoglobulin levels were found when compare to control. The T-cell population (28%) was less than half that normally found in the Indian population (65%) suggesting a potential suppression of cell mediated immunity by MIC **[14].**

Our investigation exhibit that real time detection technology using FRET probes present superior sensitivity over conventional detection methodologies for rapid diagnosis of MTB. The results obtained for the group A, B and C depicted the accuracy of the real time assay as demonstrated by the congruency in the both qualitative and quantitative results

Active TB occurs in people with apparently normal immune system and in HIV infected people before profound depletion of circulating CD4+ lymphocytes. Macrophages function as phagocytosis, but it does not kill MTB, they promote its growth, thus the predominant activator of macrophage microbicidal activity is IFN-gamma.

IFN-gamma is secreted by lymphocytes in response to Ag stimulation and has shown to be a determinant of resistance to tuberculosis in mice, disseminated TB is seen in IFN-gamma knockout mice. IFN-gamma is capable to activate the macrophages to control the growth of intracellular pathogens but unable to restrict or kill the MTB which proves that MTB interfere with cellular signal Transduction pathways that are activated by IFN-gamma and thereby avoids being killed within the Macrophages.

The Genes/Protein that are responsible for tuberculosis are , IFN-gamma, TNF-a, IL-1b, IL-6, IL-12, IL-10, IL-4

IFN-gamma is low in patients suffering from tuberculosis, both in specific and non-specific MTB stimulate. The Production of pro Inflammatory cytokines TNF-a, IL-1b, IL-6 and IL-12 are higher in patients with Tuberculosis. The Production of IL-10 remains same in patient with or without Tb.

Mechanism for the bacteria (*Mycobacterium tuberculosis*) to cause TB in humans.

The signal transduction pathway initiated by IFN-γ is becoming increasingly well characterized.

Step 1 Binding of IFN-γ to cell surface receptors results in activation of the tyrosine kinases JAK1 and JAK2, leading to phosphorylation of cytoplasmic STAT1.**[15, 16]**

Step 2 Tyrosine-phosphorylated STAT1 homodimerizes through interaction of the SH2 domain on one molecule with phosphotyrosine on another and translocates to the nucleus. In the nucleus, STAT1 homodimers activate transcription of specific genes that possess γ-activation sequences (GAS; consensus sequence is TTNCNNNAA). Human genes that contain GAS include Fcγ receptor type I (CD64), guanylate binding protein-2, class II *trans*-activator, and indoleamine-2,3-dioxygenase. **[15, 16]**

The two other pathogens have been found to inhibit IFN-γ signaling. *Leishmania donovani* inhibits IFN-γ responses by inhibiting tyrosine phosphorylation of STAT1 by JAK1 and JAK2, and human CMV inhibits responses to IFN-γ in infected fibroblasts and endothelial cells, by depleting cells of JAK kinases through degradation by proteosomes, whereas

infection with *M. tuberculosis* does not inhibit STAT1 tyrosine701 or serine727 phosphorylation, dimerization, nuclear translocation, or recognition of specific DNA sequences. **[15, 16]**

Step 3:- But infection with *M. tuberculosis* inhibits IFN-γ responses by directly or indirectly disrupting the essential interaction of STAT1α with the transcriptional coactivators CREB binding protein (CBP) and p300. The mechanism by which *M. tuberculosis* infection disrupts the STAT1α-CBP/p300 interaction remains to be elucidated, and one or more mechanisms could be responsible. First, another transcription factor could compete with STAT1 for the same binding site(s) on CBP/p300. **[16]**

2nd Mechanism :

A "Recent study" found that **activation of Jurkat cells** with IFN-α inhibits TNF-α activation of transcription of the HIV-1 long terminal repeat because STAT2 (activated by IFN-α) binds the same domain on p300 as does NF-κB (activated by TNF-α). Because Jurkat cells possess ~28,000 molecules of p300 and ~150,000 molecules of STAT2 per cell, activation of STAT2 sequestered p300 so that it was unavailable to NF-κB. **[15]**

Similarly, STAT1α and AP-1/ets have been found to inhibit one another's actions by competition for a limiting quantity of CBP. In view of these findings, *M. tuberculosis* might indirectly inhibit IFN-γ responses by activating a macrophage signaling pathway that requires CBP and/or p300 and thereby restricts the availability of these coactivators for use by STAT1α.**[16]**

Alternatively, *M. tuberculosis* could directly target the domains of either STAT1 or CBP that are involved in their protein-protein interaction. The interaction of STAT1α with CBP is mediated by binding of the N-terminal domain of STAT1α to **Cys/His-rich domain 1** of CBP and binding of the C-terminal domain of STAT1α to **Cys/His-rich domain 3**, and *M. tuberculosis* could target any of these domains on either protein to disrupt their association. **[16]**

A precedent for such a mechanism was recently reported; in addition to the well-established interaction of adenovirus E1A with p300, E1A also directly interacts with the C-terminal domain of STAT1α and blocks IFN-γ activated transcription. **[16]**

Another potential target of such a mechanism is the **N-Myc interactor protein** (Nmi). Nmi markedly stabilizes the interaction of STAT1α and CBP and thereby enhances IFN-γ responses. Therefore, interference with Nmi could yield the decrease in STAT1-CBP/p300 association that we observed in *M. tuberculosis*-infected macrophages. But still additional experiments will be necessary to determine which of these mechanisms may account for the decreased association of STAT1 and CBP/p300 observed in *M. tuberculosis*-infected macrophages. **[16]**

LAM(lipoababinomannan) is unlikely to be the sole component of *M. tuberculosis* that initiates the inhibition of IFN-γ responses.

M. tuberculosis LAM has been reported to inhibit **mitogen-activated protein** kinase (MAPK) activation in human monocytes by activating the protein phosphatase **SHP-1** (Src homology domain 2-containingtyrosine phosphatase-1). Because MAPK can catalyze the phosphorylation of STAT1α Ser^{727}, it was essential to consider inhibition of MAPK activity as a possible mechanism by which *M. tuberculosis* inhibits IFN-γ responses. Because, Firstly STAT1α is phosphorylated on Tyr^{701} and Ser^{727} to the same extent and with the same kinetics in uninfected and *M. tuberculosis*-infected macrophages makes this effect unlikely to account for inhibition of IFN-γ responses. **[17]**

An additional possible target of *M. tuberculosis* is one or more additional kinases activated by IFN-γ, such as the renaturable tyrosine kinases whose activation by IFN-γ is sensitive to inhibition by TGFß. These kinases may be sensitive to inhibition by other mediators activated by *M. tuberculosis*. **[17]**

Mean bacterial load studied in MIC-affected and non-MIC affected MTBC patients clearly states that, MIC is the major cause for increased prominence of tuberculosis in the patients. The *Mycobacterium tuberculosis* inhibits the IFN-γ responses by disrupting the interaction of STAT1α with CREB Binding Protein (CBP) and p300. CBP gene is ubiquitously expressed and is involved in the transcriptional coactivation of many different transcription factors. *M. tuberculosis* infection disrupts the STAT1α-CBP/p300 interaction. Also MTB inhibits IFN-γ responses by activating macrophage signaling pathway which restricts the availability of CBP and p300 for use by STAT1α. Also, N-Myc interactor protein (Nmi). Since Nmi stabilizes the interaction of STAT1α with CBP thereby increasing IFN-γ responses, interference with Nmi could decrease the STAT1α-CBP/p300 association in *M. tuberculosis* infected macrophages.

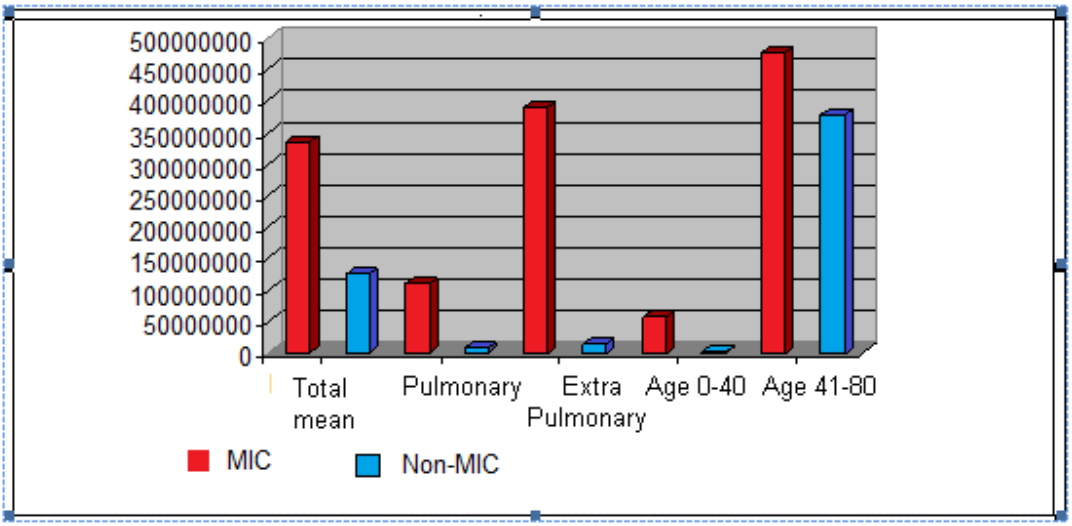
Through bioinformatics analysis determine the how MIC can disrupts binding sites of STAT1α and CBP/p300. Binding affinity of MIC on STAT1α and CBP/p300 binding sites will be studied by Glide molecular docking program of Schrodinger software which will give the docking score and on the basis of score , binding sites can be determined. This can be compared with STAT1α-CBP/p300 binding affinity in non-MIC affected patients which explain about the mean bacterial load less in non MIC patients.

Table 1: Mean Bacterial load of different groups of Methyl Isocyanate (MIC) affected and Un affected Patients

S. No	Groups	Mean Bacterial Load of MIC patients	Mean Bacterial Load of Non MIC patients
1	Total Patients	$3.34x10^8$	$1.3x10^8$

2	Pulmonary	$2.09x10^8$	$8.7x10^6$
3	Extra pulmonary	$3.65x10^8$	$1.4x10^8$
4	0-40 age	$5.7x10^7$	$9.9x10^5$
5	41-80 age	$4.7x10^8$	$3.7x10^8$

Graph of Bacterial Load in MIC affected and unaffected patients in different groups



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