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Virulence in isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis* from south India

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ABSTRACT

Isoniazid, is the only antituberculous drug for which the relation between lack of virulence and acquisition of resistance was associated. INH-resistant mutants were shown to contain defective *katG* gene. Classical studies showed that INH-resistant south Indian isolates have lower virulence in guinea pigs and higher susceptibility to H_2O_2. It is of interest to assess the virulence in south Indian clinical mutants of KatG (catalase-peroxidase enzyme) associated with INH resistance. Five INH-resistant clinical isolates were selected on the basis of mutation in *kat*G gene. Mutant isolates were used for infecting macrophage cell line (THP-1) and for the assessment of enzyme activity. In comparison to control H37Rv, Ser315Thr mutant exhibits similar virulence and reduction (4% and 17%) in catalase (C) and peroxidase (P) activity. For the mutants, Ser315Iso and Ser315Arg, the virulent nature was slightly reduced with 3% and 2%, and significant reduction was observed in the C (50%; 47%) and P (36%; 43%) activity respectively. Ser315Asn indicates 4% reduction in virulence with significant reduction in C (56%) and P (64%) activity. Asn138Ser mutant displayed low-level virulence, CP activity showed 47% and 56% reduction. The results indicate that similar level of virulence in all the mutants except Asn138Ser which showed relatively low-level whereas a significant decrease in C and P activity was observed in all mutants except Ser315Thr. The study suggests despite the fact that all mutants do not compromise survival or virulence yet provides INH resistance.

1. Introduction

Tuberculosis (TB) remains as a major cause of mortality worldwide, the emergence of multi-drug resistant (MDR) TB defined as strains which are resistant to the two most potent anti-TB drugs; isoniazid (INH) and rifampicin (RIF) have caused a deep concern globally. Presently, the condition is still complicated by the outbreak of extensive drug resistant (XDR-TB) defined as MDR-TB that is resistant to second-line TB drugs- fluoroquinolones and at least one of three injectable aminoglycosides-Capreomycin, Kanamycin or Amicacin. Drug resistance in TB is essentially a potential threat to the TB control programmes. However, isolates of *M. tuberculosis* resistant to INH are seen with increasing frequency (1 in 10^6) compared to other drugs (Nachega and Chaisson, 2003). The association between acquisition of resistance and lack of virulence was concerned with INH alone. The link between catalase and virulence was first recognized by Middlebrook, who showed that INH-resistant (INH^r) isolates of *M. tuberculosis* were catalase deficient and markedly attenuated in guinea pigs (Middlebrook and Cohn, 1953). INH^r mutants selected *in vitro* frequently lose *katG* gene entirely.

Mycobacteria are intracellular bacterial parasites, which survive and proliferate inside the

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macrophages for long periods of time. As mycobacterial survival in macrophages is required for virulence, a great deal of effort has been focused on identifying the genetic and physiologic determinants of intracellular survival and growth. A number of factors, among them catalases, peroxidases, and superoxide dismutase have been suggested as agents permitting mycobacteria to overcome the intracellular defences of macrophages (Bartos et al., 2004).

The physiological role of KatG is protective, combating the low pH found during the "oxidative burst" in human phagocytes, where liberated O_2 radicals are converted to H_2O_2 within the phagosome. KatG enzyme activity eliminates this via a "deceptively simple reaction." The precise role of KatG in *M. tuberculosis* virulence has not been elucidated. One possibility is that KatG is required to catabolize the exogenous peroxides generated by the phagocyte oxidative burst, or peroxynitrite (ONOO) produced by the interaction of O_2^- and nitric oxide (NO). However, KatG might also be essential for detoxification of endogenous peroxides generated by bacterial respiration of nonfermentable substrates such as fatty acids, which might be the major carbon substrate used by *M. tuberculosis* in persistently infected mice (McKinney et al., 2000).

Studies from all over the world have produced conflicting results. One school of thought suggests that KatG is required for protection against oxygen free radicals within the macrophage (Manca et al., 1999) hence KatG is considered as a virulence factor. Studies relating to the strains of *M. tuberculosis* with defective *katG* gene lacks virulence in a variety of animal models (Middlebrook and Cohn, 1953; Li et al., 1998; Pym et al., 2001; Wilson et al., 1995), whereas Jackett et al., (1978) and O'Brien et al., (1991) have observed no correlation between loss of *katG* gene activity and virulence of *M. tuberculosis* in mice and guinea pigs have observed.

The availability of KatG mutant organisms has facilitated investigations of the role of CP in the virulence of *M. tuberculosis*. Contrary to previous perceptions, a mechanism (point mutation in *katG*) of INH resistance would not usually be associated with a large reduction in virulence. Instead, the strain with this mutation retains virulence in a macrophage model of TB while displaying a clinically significant level of resistance to INH. This is compatible with the observation that the KatG Ser315Thr (S315T) mutant produces a functional CP with enzymatic activities comparable to the WT protein. At least 50% of all INH^r clinical isolates of *M. tuberculosis* harbour the S315T mutation, and a much higher percentage do so if one considers only strains with clinically significant levels of resistance. The high frequency and lack of attenuation associated with the S315T substitution mean that the majority of INH^r clinical isolates will be virulent. Thus, the conventional view that INH^r strain of *M. tuberculosis* are of low virulence, prevalent since Middlebrook's original description of attenuated, catalase-negative, INH^r isolates of *M. tuberculosis* (Middlebrook, 1953; 1954), needs to be modified. It is also significant that the S315T substitution is particularly associated with MDR strains of *M. tuberculosis* (Marttila et al., 1996; Piatek et al., 2000). MDR strains will exert a cumulative effect on virulence. Mutations such as S315T, which minimize the cost of antibiotic resistance, may therefore favor the emergence of transmissible MDR strains.

In contrast to S315T mutant, production of the less stable Thr275Pro mutant by *M. tuberculosis* is associated with greatly reduced virulence in the macrophage model of disease, undetectable CP activity and high-level INH resistance (Pym et al., 2002). Also, in one of the report, Thr275Pro mutant expressed low levels of enzymatic activity and failed to persist in guinea pig spleen, although they did survive in mouse tissues (Li et al., 1998). Characterization of another mutant Ala139Val demonstrated that there was no significant difference in virulence and catalytic activities between the mutant and WT protein (Pym et al., 2002).

In human monocytes *in vitro*, the ability of *M. tuberculosis* to withstand killing by exogenously added or endogenously stimulated H_2O_2 is important for bacillary survival. The KatG-negative (INH^r) *M. tuberculosis* appear as less virulent in humans but still can cause disease in humans during *in vivo* oxidative burst. This may be due to insufficient levels of ROI in the infected lung. The extent of the oxidative burst present in macrophages *in vivo* is unknown. The levels of ROI achieved within the granuloma may differ from individual to individual (Manca, 1999). INH^r mutant strains of *M. tuberculosis,* which have no detectable KatG

activity, acquire a compensatory mutation resulting in an up-regulation of expression of AhpC. It has been suggested that this protein confers protection against H_2O_2 mediated damage even in the absence of adequate KatG activity, thus promoting survival of the organism in the environment of the phagocyte oxidative burst (Sherman et al., 1996).

Virulence of *M. tuberculosis* strains has traditionally been assessed in terms of the ability of bacilli to replicate within specific organs of mice and guinea pigs following aerosol infection, intramuscular injection as mentioned in the above reports. Such studies are time-consuming and expensive. Furthermore, differences among strains of *M. tuberculosis* in the course of infection and in the protective mechanisms required for containment of the organism may limit the applicability of these studies to understand the attributes of virulence. Moreover, virulence studies performed way back in 1960s showed that south Indian isolates have lower virulence in guinea pigs and higher susceptibility to H_2O_2. In the light of which, interest is shown to explore the virulence in clinical mutants of KatG associated with INH resistance in south Indian isolates using macrophage cell line (THP-1). Although previous studies have assessed the intracellular growth of selected INH^r clinical isolates of *M. tuberculosis* in mouse models and human monocytes (Pym et al., 2002; Manca et al., 1999). The relative role of the KatG mutants, which occurs at codon 315 in mediating the virulence, has not been previously evaluated. The present study represents the first to examine the panel of INH^r clinical mutants of KatG such as Ser315Iso (S315I), Ser315Arg (S315R), Ser315Asn (S315N) and Asn138Ser (N138S) in addition to the already reported S315T mutant.

2. Materials and Methods

2.1. Clinical isolates

In this study, five INH^r clinical isolates of *M. tuberculosis* were selected from patients of south India (20-60 years) including both the sexes from 2006-2009. Drug susceptibility testing (DST) was performed at National Institute for Research in Tuberculosis (NIRT), Chennai, India. DST was performed using Lowenstein–Jensen (LJ) medium and INH susceptibility was determined by minimum inhibitory concentration (MIC) method. Out of five, two of them were MDR, three were INH mono-resistant isolates and a laboratory reference strain of *M. tuberculosis*, H37Rv, was used as the control [Table 1]. A total of 105 isolates were used for mutational analysis (data in communication) of which, five isolates were chosen after automated DNA sequencing with genome having mutations at codon 315 such as ACC-Thr, AAC-Asn, ATC-Iso, CGC-Arg and AGC-Ser at codon 138 and the control having a wild type (WT) codon AGC-Ser at 315. These strains were used for infecting THP-1 cell line.

2.2. Amplification

Amplification was performed in the isolated genomic DNA using the mixture containing 1 µl of forward and reverse primers (10 pmol) each, 6 µl of deoxyribonucleoside triphosphates (dNTP) mix (2.5mM), 2.5 µl of 10X PCR buffer, 10-50 ng of template genomic DNA and 1 U of *Taq* DNA Polymerase (Amersham Biosciences, UK). The amplification was performed in a thermal controller (MJ Research, USA) with 30 cycles (1 min at 95°C, 30 sec at 63°C and 1 min at 72°C, followed by a final extension step at 72°C for 10 min). The primers of *katG* gene forward sequence (5'-AAACAGCGGCGCTGGATCGT-3') and reverse sequence (5'-GTTGTCCCATTTCGTCGGGG-3') were used to generate a 209-bp fragment containing the S315T codon. The amplicons were purified using GFX COLUMN (Amersham Biosciences, UK) according to the manufacturer's instructions.

2.3. DNA Sequencing

Sequencing of the amplicon was carried out using an automated DNA sequencer (ABI Prism 310 Genetic Analyzer-Applied Biosystems, USA), using the above mentioned primers and the Bigdye terminator sequencing kit (Applied Biosystems). To 4 µl of the Terminator ready reaction mix, 1 µl of the amplified fragment (2–3 ng) and 1 µl of the primer (10 pmol/ µl) were added and the volume was made up to 20 µl using deionised water. The reaction mixture was subjected to cycle sequencing. The samples were vortexed and spun, then heated at 95°C for 2 min and immediately chilled on ice. They were vortexed and spun again and placed on

ice, to load onto the DNA sequencer. The GenBank accession number for *katG* is X68081. The data obtained was compared with sequences from the database EMBOSS using the alignment tool via http://www.ebi.ac.uk/emboss/align/.

2.4. Growth and maintenance of tubercle bacilli

All the strains were grown to mid log phase in Middle brook 7H9 medium with 0.05% Tween-80 instead of glycerol. The suspension was frozen in aliquots with 20% glycerol at –70°C until use.

2.5. Single cell suspension

For infection, bacterial aliquots were thawed, centrifuged for 15 min at 3000 rpm and 23°C. The bacterial pellet was suspended in 500 µl of RPMI and vortexed along with glass beads for 30 sec. The suspension was made to a final volume of 5 ml with RPMI medium. To disrupt the bacterial aggregates, the medium was syringed several times by repeatedly forcing it out of 1 ml insulin syringe. The suspension was centrifuged at 700 rpm for 2 min, or left to stand for 5-10 min. The top layer represents the single cell suspension. Small aliquots of the suspension were subjected to counting. Counting of bacilli was done in a Thoma counting chamber: Where, 2×10^7 is the correction factor, (X = no. of squares counted). For counting by Colony Forming Unit (CFU) aliquots were serially diluted to five fold in sterile Phosphate Buffer Saline (PBS) and plated on 7H11 agar plates. After incubation for three weeks at 37°C, CFU were calculated. CFU was calculated using colony count x dilution factor x N (N = spotting volume).

2.6. Growth and maintenance of THP-1 cell line

THP-1 cell line (human monocytic leukemia cell line) obtained from National centre for cell science (NCCS), Pune. The cells were propagated in the maintenance media. Its doubling time was approximately 18 – 24 hrs. Contamination of the media with bacteria or fungus can be better assessed with its apparent turbidity and also by the grape like clusters formation under a phase contrast microscope. The pale yellow colour change of the media signifies its acidic nature. The cell lines were maintained in endotoxin free Roswell Park Memorial Institute (RPMI) medium containing 10% heat inactivated Fetal Calf Serum (FCS) with antimycotic antibiotic cocktail at 37°C in a humified 5% CO_2 atmosphere.

2.7. Counting of cell line

The grown cultures of THP-1 cells were pelleted down at 2000 rpm for 10 min in falcon and supernatant was decanted. The cells were washed with RPMI and 10% FCS twice (as cells are grown in antibiotic media). The 5 ml of fresh RPMI was added to pellet and centrifuged at 2000 rpm for 10 min. Then the pellet was dissolved in the required volume depending upon the experiment. For counting, 5 µl cells were taken with 85 µl of plain RPMI and 10 µl of 0.4% trypan blue dye (1:20 dilution). Cells were mounted in Neubauer chamber immediately.

2.8. Trypan blue exclusion test

The degree of structural integrity of the cell membrane is used as the parameter to determine cell viability. Trypan blue as a dye cannot enter cells through an intact membrane and therefore stains only cells, which have punctured membranes. Dead cells stain blue, while live cells exclude trypan blue. The counted monocytes were differentiated into macrophages by the addition of 10nmol of Phorbol 12-myristate 13-acetate (PMA) and incubated overnight, the activated cells (macrophages) were observed in phase contrast microscope the following day.

2.9. Infection

For the infection experiments, multiplicity of infection (MOI) of 1:10 (Macrophage Mycobacteria) was used. One million THP-1 cells in a well were seeded (after counting) in the presence of 1 ml of RPMI and 10% FCS in 24-well plate. After differentiation of cell line, ten million tubercle bacilli (INH^r clinical mutants of KatG) were added uniformly to all the wells. Negative controls include plain THP-1 cells and bacilli in two wells separately. The cells were incubated at 37°C in a humified 5% CO_2 atmosphere for required period of days and subjected to lysis.

The cells were lysed after removal of the media and washed thrice with 1ml of sterile PBS to remove extracellular bacilli; the antibiotic amikacin was also used for one of the experiment parallel to washing. Then 200 µl of ice cold PBS was added followed by the addition of 60 µl of 0.02% of SDS. To remove the intracellular bacilli from the cell line, the contents were scrapped nicely with the micropipette tip and the scrapped content constitutes the lysate. The lysates were serially diluted to five fold in sterile 450 µl of PBS and plated on 7H11 agar plates. After incubation for three weeks at 37°C, CFU were calculated.

2.10. Staining

Kinyoun's: The coverslips with adherent mycobacteria and macrophages were covered with 1% carbol fuschin stain for 10 min. Excess stain was then carefully washed with distilled water. Coverslips preparations were then decolourized with 1% acid alcohol until colour disappears. Cells were counter stained with 2% methyl green for 5 min and the coverslips washed in distilled water. The stained coverslips preparations were air dried and mounted on a glass slide (Kinyouns, 1915). The cells were observed under microscope using 100X lens.

Fluoresence Microscopy (FM): Freshly prepared filtered 0.3% auramine phenol was poured over the coverslips with adherent mycobacteria and macrophages fixed to the slides and left for 7 – 10 min. The slides were carefully washed with distilled water in a Petri dish. 1% acid alcohol was then used for decolourization for 1-2 min. Then the slides were gently rinsed with distilled water and counter stained with 0.1% potassium permanganate for 30-40 sec, followed by gentle washing with distilled water. The stained coverslips preparations were air dried and mounted on a glass slide (Freiman and Mokotoff, 1943). The cells were observed under microscope using 40X lens.

2.11. Assay of mutants

The WT H37Rv and INH^r clinical mutants of KatG were grown on LJ slants for 2 weeks and then a loopful of culture from the slant was inoculated in 20 ml of Sauton media (ST). The cells were incubated at 37°C for 2 weeks without contamination. Scaling up was done by transferring 20 ml of the grown culture to 200 ml of ST and incubated until it reaches exponential phase and then it was harvested. The culture was centrifuged at 3000 rpm for 15 min and washed with PBS thrice. According to the cell mass (wet weight) the required volume of cocktail of protease inhibitor was added, it was sonicated for 30 sec with four burst by keeping on ice. The supernatant was transferred and maintained at –20°C as crude lysate where it as stable for several months. (Loewen et al., 1986).

2.12. Catalase and Peroxidase assay

Diluted samples of enzyme were taken in 1 ml of 50 mM potassium phosphate buffer pH 7.0. Reading was recorded every 2 min followed by addition of 10mM H_2O_2 whereas the blank constitutes the buffer and H_2O_2 without enzyme (Devi et al., 1975). Catalase activity as U/ml was determined from the slope of the plot representing oxygen evolution. Catalase activity was assayed spectrophotometrically by monitoring the decrease in H_2O_2 concentration at A_{240} (ε_{240} = 0.0436 mM^{-1} cm^{-1}).

Peroxidase activity was determined spectrophotometrically by ABTS method (Smith et al., 1990) with minor modifications. Assays were carried out in 1 ml final assay volumes containing 10 mM H_2O_2, 2.5 mM of 2,2- azinobis (3 ethylbenzothiazolinesulfonic acid (ABTS) in 50 mM potassium phosphate pH 7.0. Aliquots of the appropriately diluted enzymes were added to initiate the reaction. Peroxidase activity was determined by measuring the oxidation rate of ABTS at A_{405} (ε_{405} = 36.8 mM^{-1} cm^{-1}).

2.13. Statistical analysis

The mean difference, standard deviation (SD), P value ($P < 0.05$ was considered significant by Students t-test [two tailed, unpaired]) was obtained using Graph Pad Prism software V-5.0.

3. Results

The relationship between INH resistance and virulence at the cellular level was explored by infection studies with the help of THP-1 cell line.

For experimental purposes, cells were washed twice to remove antibiotics in a 24-well plate at a density of 1million/mlRPMI/well. The total number of cells in the suspension was enumerated in a Neubauer chamber and the viability was determined by trypan blue dye exclusion method. The cell line was differentiated into macrophages by the addition of PMA before infecting with tubercle bacilli (Figure 1A). The bacilli after infection are shown in FM (Figure 1B) and Kinyoun's staining (Figure 1C and 1D).

The bacilli were enumerated by CFU count before infection intracellular viability of the tubercle bacilli was assessed on 0^{th}, 4^{th} and 7^{th} days as CFU obtained from lysed macrophages (Figure 2A). The catalase and peroxidase activity were found in the crude cell extracts of all the mutants along with the WT. During the course of virulence investigation, there was no significant difference observed among the isolates compared to the virulent standard H37Rv except the isolate N138S which showed slightly significant ($P < 0.05$) low-level virulence (Figure 2B). The KatG protein was implicated early in studies of mycobacterial pathogenesis because of the availability of mutants, which had lost KatG function through the acquisition of INH resistance. Even with these mutants, the relative contribution of the enzyme to mycobacterial survival within the host phagocyte has remained unclear.

Table 1. Intracellular viability and enzymatic activity of KatG mutants with MIC of INH

Clinical isolates	DST	MIC [mg/ml]	KatG mutants	Genotype	CFU ± SD	Catalase activity ±SD	Peroxidase activity ±SD
H37Rv		<0.2	S315	AGC	6.33±0.14	0.54 ±0.03	0.49 ± 0.012
RF10773	MDR	1	S315T	ACC	6.27±0.16	0.52± 0.007	0.41 ± 0.025
003630	MDR	5	S315I	ATC	6.20± 0.07	$0.30±0.03^{b}$	$0.26 ± 0.017^{b}$
DP0085	INH	>5	S315R	CGC	6.21±0.16	$0.35±0.04^{b}$	$0.28 ± 0.034^{b}$
00993	INH	>5	S315N	AAC	6.12±0.1	$0.24 ±0.09^{b}$	$0.18 ± 0.018^{b}$
988412	INH	>5	N138S	AGC	$6.08±0.02^{b}$	$0.29±0.04^{b}$	$0.22 ± 0.025^{b}$

Results are representative of three activities and CFU (0^{th}, 4^{th} and 7^{th} day), b= $P < 0.05$

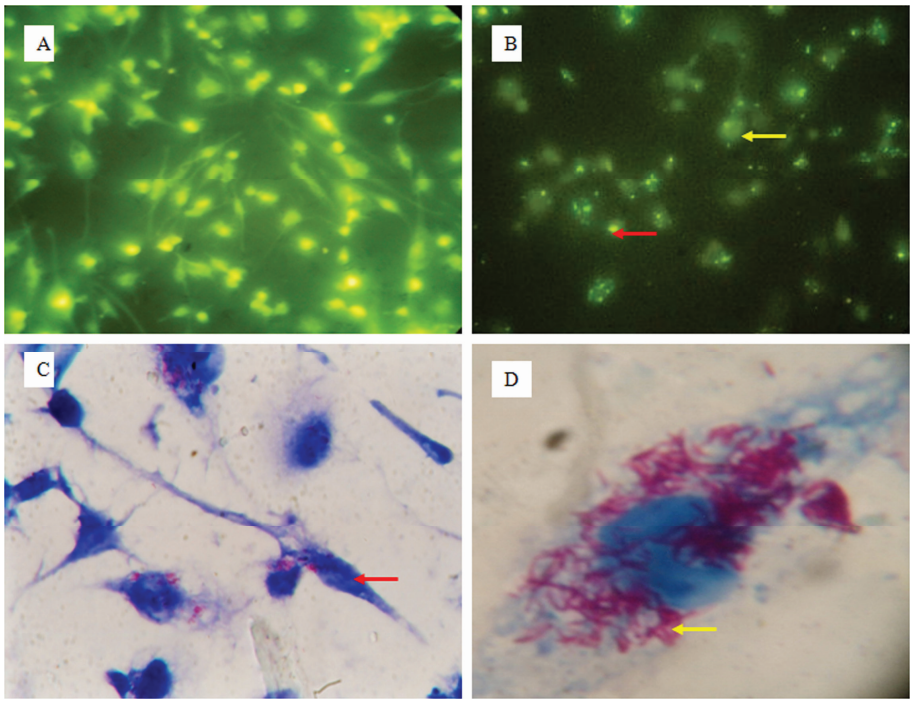

Figure 1. A and B: FM staining showing Differentiated cells (1A) Infected cells (1B) Magnification at 40X. 1C and 1D: Kinyoun's staining showing infected cells. The yellow arrow showing tubercle bacilli and the red arrow showing macrophages Magnification at 100X

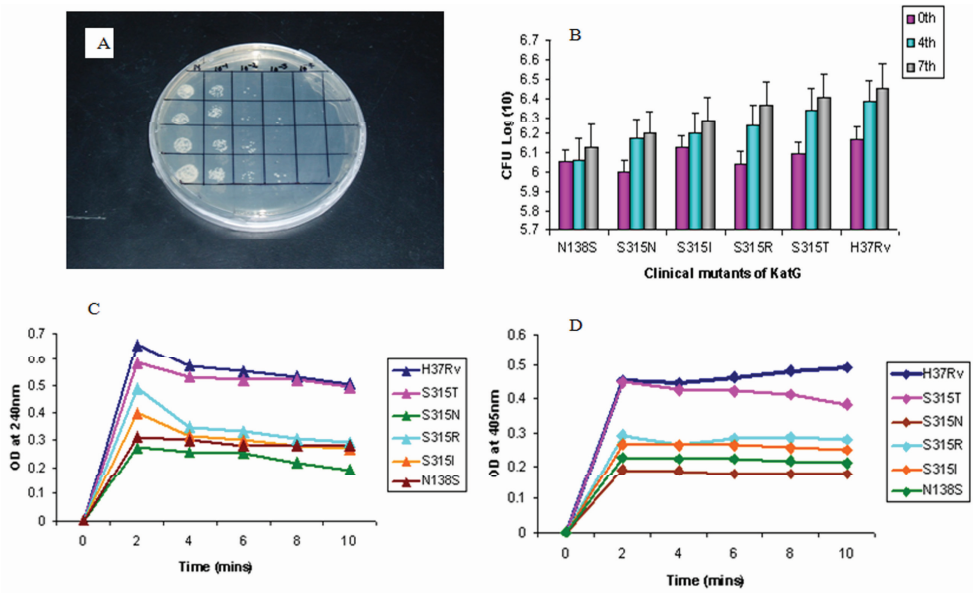

Figure 2. A: CFU of cell lysate B: Intracellular growth displayed by KatG mutants C: Catalase activity D: Peroxidase activity of KatG mutants

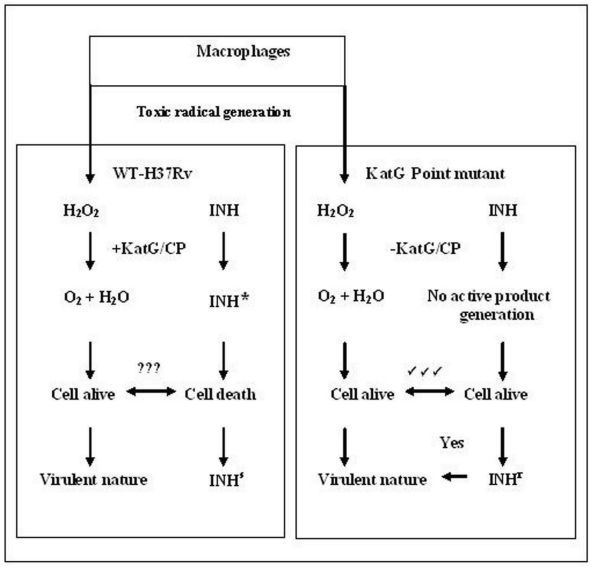

Figure 3. Relationship between INH^r and virulence mediated by KatG/CP in WT and Mutant strains of *M. tuberculosis* (Proposed based on KatG point mutants including our study).

INH* = Active form of INH

??? = How these contrary events do occur in the same cell

✓ ✓ ✓ = This situation is possible in a cell representing INH^r strains are relatively virulent

4. Discussion

Survival of *M. tuberculosis* within the host macrophage phagosome requires that the bacilli be capable of resisting the normal microbicidal mechanisms of potent leukocytes. Some of the inconsistency in the published studies may be attributable to the use of a wide variety of model systems for evaluating virulence in which the actual effector molecules involved in mycobacterial killing

may differ e.g., nitric oxide synthase-mediated killing in murine macrophages (Chan et al., 1992) versus ROI-mediated killing in human mononuclear phagocytes (Laochumroonvorapong et al., 1997).

The potential of INH^r clinical isolates to infect THP-1 cells, which is the earliest step in *M. tuberculosis* infections, was explored. The interaction between bacilli and macrophage starts with binding of bacterium to cell surface with subsequent internalization/phagocytosis, which involve the interaction of *M. tuberculosis* ligands and macrophage receptors.

In an attempt to characterize the virulence of INH^r clinical isolates *in vitro*, we assessed the levels of activity and the growth rate by five such isolates following THP-1 infection. The findings were compared with WT H37Rv on the basis of genotypic content of the isolates. The major concern with this kind of studies is the validity of this *in vivo* experimental model used in order to extrapolate data to humans.

In this study, five genotyped INH^r clinical isolates of *M. tuberculosis* strains were used. The virulence status of various strains was assessed by infecting the macrophage cell line. The rationale for mutant selection is the first of which, the S315T isolate, harbours the most frequently occurring mutation while the second, third, fourth and fifth isolates had S315N, S315I, S315R and N138S mutation found occasionally in INH^r clinical isolates. Thus, the study includes all the mutations which occur at codon 315 excepting S315G as was stated by Ramaswamy and Musser (1998), in addition to the most frequently occurring mutation Ser to Thr at codon 315, Ser also has been reported to be mutated to Asn, Iso, Arg, and Gly. Among the KatG defective mutants, the most frequently encountered INH resistance-conferring mutation; KatG S315T found to result in near-normal CP activities in our study as already reported (Rouse et al., 1996; Pym et al., 2002; Joseph and Morris, 2003; Wengenack et al., 1997). The mutant also exhibits near-normal levels of virulence compared to the WT H37Rv, while conferring resistance to INH as previously reported (Pym et al., 2002).

The N138S clinical mutant displayed low-level virulence and CP activity. The N138S recombinant mutant catalytic activity has been described previously by Joseph and Morris, who showed that the mutant had substantially reduced CP activity yet, remained highly sensitive to INH (Joseph and Morris, 2003). The reduction in the activity of this mutant may be due to interference with substrate access at the active site of KatG. The modeling studies of KatG reported that a substitution which occurs near the catalytic site of the enzyme influences its activity (Rouse et al., 1996).

The clinical mutant S315N presented here showed a small reduction in virulence and significant reduction in CP activity. However a report (Wei et al., 2003) documented reduced catalytic activity of this recombinant mutant and its inability to activate INH for the formation of InhA inhibitor. For the mutants S315I and S315R the virulent nature was slightly reduced with significant reduction in the CP activity in comparison to control WT H37Rv.

TNhe investigation of CP activities relative to the WT H37Rv showed that N138S mutant caused 44% and 56% ($P < 0.05$) reduction in C and P activity respectively, while the WT H37Rv (S315) and the S315T mutant showed near-normal activity in the extracts. Activities reductions of 50%, 47% and 36%, 43% were detected in the S315I and S315R mutants ($P < 0.05$) respectively. For the S315N mutant 56% and 64% ($P<0.05$) decrease in C and P activity were observed. Therefore all the mutants excepting S315T displayed significant decrease in enzyme activity and the degree of variation was more for S315N mutant compared to others (Figure 2C, 2D and Table 1).

Our data conclusively showed that there was no significant difference among the strains related to the virulent standard although this difference was not maintained by one of the isolate N138S showing slightly significant ($P < 0.05$) low-level virulence. All the clinical isolates reached higher bacillary concentrations by day 7 than day 4. To study the intracellular survival of laboratory and clinical strains, number of CFU was determined on 0, 4 and 7 day, via infection in differentiated THP-1 cells. The analysis of the data indicates that all mutants showed a gradual increase in CFU from day 0-7. On day 7, WT infected cells showed increase in bacillary load compared to other mutant strains. The infection rate (CFU) of WT H37Rv was 6.33 expressed as log CFU (per 10^6 macrophage). The mean intracellular growth of WT at zero day was 6.17. Differences in the intracellular growth

were apparent on day 4 and 7 with 6.38 and 6.45 as CFU respectively (Figure 2B and Table 1).

Study of virulence in drug-resistant clinical strains in the experimental model of TB infected through aerogenic exposure has already been performed and catalase activity did not seem to have a meaningful impact on the degree of virulence exhibited by the strains (Cardona et al., 2003). However, in this study an extent of inverse relationship between the virulence and activity imparted by the isolates was obtained. The analysis of infection rate (CFU) suggests the nearly virulence nature of the isolates whereas the rate of enzyme activity suggests the isolates are less virulence.

The findings indicate that, despite its laboratory cultivation for nearly a century, H37Rv displays an *in vitro* phenotype that is at least as robust as and significantly more reproducible than that of a variety of INH^r clinical isolates of *M. tuberculosis,* suggesting that H37Rv should remain a preferred reference strain for many types of *in vitro* studies.

Although four of the strains exhibited high-level INH resistance of >5 mg/L, based on the observations it was emphasized that INH^r clinical isolates from south India are nearly virulent as WT H37Rv. This is against the assumption that INH^r *M. tuberculosis* isolates from south India are inherently less virulent than fully susceptible organisms (Mitchison et al., 1960 and 1963). Indeed, the findings suggest that the genetic background of south Indian isolates (point mutants not deletion mutant) may contribute to the virulent nature. Manca et al. (1999) has reported that KatG-negative (INH^r) *M. tuberculosis* should be less capable of withstanding the *in vivo* oxidative burst in human mononuclear phagocytes and therefore less pathogenic in humans. Nevertheless, KatG-negative (INH^r) mycobacteria can cause disease in humans. This possibility is partly supported by this study that unlike most resistance-conferring mutations, N138S was found to result in reduced CP activities and levels of virulence causing disease while also conferring resistance to INH because defective KatG unable to activate the drug leads to resistance and less efficient in combating the oxidative burst, hence pathogenic. Contrary to previous perceptions (Middlebrook, 1953 and 1954) of avirulent nature of INH^r strains, S315T related mechanism of INH resistance is not usually be associated with a large reduction in virulence and is an exception to the rule that antibiotic-resistance-conferring mutations carry a significant fitness cost. It is also pertinent that the S315T substitution is particularly associated with MDR strains of *M. tuberculosis*. This has important implications for the transmission and control of MDR-TB (Pym et al., 2002).

In summary, the phenotype of KatG mutants presented here is less severe than that described previously (Li et al., 1998; Pym et al., 2001) for *katG* gene deleted strains of *M. tuberculosis.* The mutants analyzed in those studies contained large chromosomal deletions encompassing *katG* and flanking genes. KatG deletion mutants (null mutants) was not included in our study, which otherwise would have showed decreased in virulence. The reason for the retainment of virulence in our study could be to the occurrence of point mutations alone in *katG* gene, which does not disturb the virulence nature significantly. Further the KatG protein was not the sole determinant of virulence in tubercle bacilli as there are other virulence genes (Li et al., 1998) (Figure 3).

From this experiment it can be concluded that KatG-proficient strain H37Rv, and all the clinical mutants grew rapidly, suggesting that its virulence was not impaired in INH^r clinical isolates. However *M. tuberculosis* strain H37Rv provided more reproducible infection than the clinical isolates. Thus, the results obtained in this study, fits into a picture where in all these mutants does not compromise survival or virulence yet provides INH resistance.

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