

BASIC RESEARCH



Studies completed:

Evaluation of the diagnostic potential of RD-1 encoded CFP-10 antigen in TB

Background:

The 38kDa (Rv0934) has been extensively used in serodiagnosis, because it is species-specific. The use of 38kDa in serodiagnosis has yielded a sensitivity of 60 per cent in our laboratory and also in other studies. There is a consensus in the field of immunodiagnosics that multiple antigens must be used to enhance the sensitivity and that more and more species specific antigens are to be added to the 38kDa.

Aim:

To study the ability of species-specific antigen Culture Filtrate Protein -10 (CFP-10) to enhance sensitivity, especially when combined with the species-specific antigen 38 kDa.

Methods:

ELISA for antibody estimation (IgG and IgA) was carried out in the following groups:

Disease groups:

1. Smear and culture positive patients with pulmonary TB (S+C+) (n = 262)
2. Smear negative and culture positive patients (S-C+) with pulmonary TB (n = 60)
3. Smear and culture negative, but radiologically diagnosed cases (S-C-) (n = 186)

Control groups:

1. Normal Healthy Subjects (NHS) (n = 160)
2. Other lung diseases (n = 76)
3. Disease control (n = 20)

Results:

Antibody levels of class IgG, IgA and combination, for the two polar groups, S+C+ and NHS for 38kDa and CFP-10 are shown in Table 10. With 38kDa antigen, out of 262 S+C+ TB sera, 158 were positive for IgG, giving a positivity of 60.3 per cent (S+C+). In the case of CFP-10, of 262 sera (S+C+), 133 were positive for IgG yielding a positivity of 50.8 per cent.

Among 160 control sera (NHS), four were positive for 38kDa giving a specificity of 97.5 per cent and three were positive for CFP-10 giving a specificity of 98.2 per cent. Some sera showed positivity for IgA, even though negative for IgG. The addition of IgA to IgG enhanced the sensitivity. Therefore, subsequent results were



expressed as IgG+IgA positivity. The sensitivity (G+A) of 38kDa in S+C+, S-C+ and S-C- cases were 76 per cent, 57 per cent and 76 per cent respectively. The values for CFP-10 were 71 per cent, 67 per cent and 45 per cent (Table 11). The antibody response to each antigen was complementary to the other. The use of RD1-encoded specific antigen CFP-10 enhanced the sensitivity of 38kDa. The combined sensitivity was 98 per cent in S+C+ and 78 per cent in S-C+, the highest sensitivity obtained in our laboratory with combination of antigens. Therefore this antigen promises to be a good candidate antigen for serodiagnosis.

Table 10: Sensitivity / Specificity in polar groups

Isotypes	38kDa antigen				CFP-10 antigen			
	S+C+ (n=262)		NHS (n=160)		S+C+ (n=262)		NHS (n=160)	
	No. +ve	%	No. +ve	%	No. +ve	%	No. +ve	%
IgG	158	60.30%	4	97.50%	133	50.80%	3	98.10%
IgA	88	33.60%	4	97.50%	109	41.60%	3	98.10%
IgG+IgA	199	75.90%	8	95%	186	71%	6	96.20%

Table 11: Positivity by combination of antigens

Category	38 kDa		CFP-10		38 kDa + CFP-10	
	IgG + IgA		IgG + IgA		IgG + IgA	
	No. + ve	% sen.	No. +ve	% sen.	No. +ve	% sen.
S+C+ (N=262)	199	75.9	186	71	257	98.09
S-C+ (N=60)	34	56.7	40	66.7	47	78.3
S-C- (N=186)	142	76.3	83	44.6	146	78.4
Other lung diseases (N=76)	2	97.4	3	96.1	5.	93.43
Disease control (N=20)	1	95	1	95	2	90
NHS (N=160)	8	95	6	96.2	14	91.2

% sen. Percentage sensitivity
 S+C+ Smear positive; Culture positive group
 S-C+ Smear negative; Culture positive group
 S-C- Smear negative; Culture negative group
 NHS Normal Healthy Subjects



Regulatory role of HLA-DR2 on macrophage phagocytosis and perforin positive cells in pulmonary TB

Background:

The study is part of the ongoing project on the role of HLA-DR2 on immune functions in pulmonary TB.

Aim:

To understand the role of HLA-DR2 gene on innate immune functions such as macrophage phagocytosis with live *M. tuberculosis* and perforin positive cells in pulmonary TB.

Methods:

The study subjects included 70 pulmonary TB patients and 70 normal healthy volunteers. DNA typing of HLA-DR, enumeration of *ex vivo* perforin positive cells by flowcytometry, macrophage phagocytosis with live *M. tuberculosis* was carried out on patients and normal subjects.

Results:

Macrophage phagocytosis:

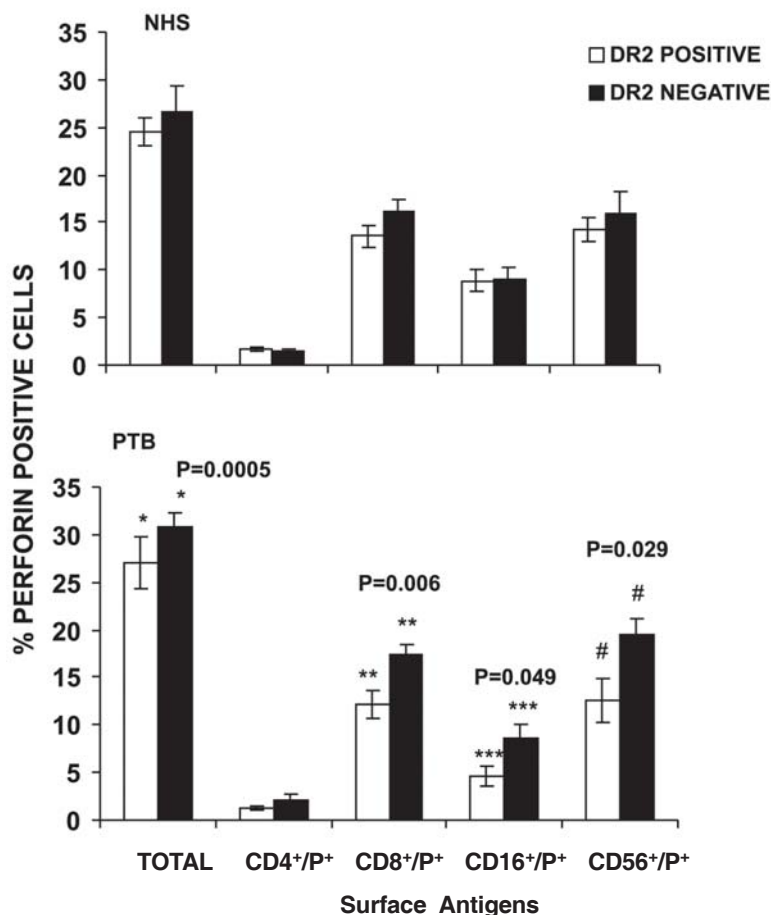
A trend towards an increased percentage macrophage phagocytosis was observed in normal subjects with HLA-DR1, as compared to subjects with non-DR1 antigens ($p=0.07$). Significantly decreased macrophage phagocytosis was observed in normal persons with -DR3 ($p=0.04$) and -DR10 ($p=0.0001$) antigen than -DR3 and -DR10 negative individuals. Whereas, no such difference in the macrophage phagocytosis was observed in pulmonary TB patients. Moreover, analysis on the phagocytosis of -DR1, -DR3 and -DR10 positive normal subjects revealed a trend towards an increase in phagocytosis with DR1/DR2 combination and a decrease with DR2/DR3 (DR 1/2 vs. DR 2/3) ($p=0.03$) and DR2/DR10 combinations.

Ex vivo perforin positive cells:

Significantly decreased overall intracellular perforin positive cells were observed in HLA-DR2 positive TB patients, than non-DR2 group ($p<0.001$). Further analysis on intracellular perforin positive cells with various surface markers, revealed a significantly low perforin positive CD8 ($p<0.01$) CD16 ($p=0.049$) and CD56 ($p=0.029$) cells in HLA-DR2 positive pulmonary TB patients than non-DR2 patients (Fig.3). However, no such decrease was observed in normal subjects with and without -DR2 antigen.



Fig.3: Intracellular perforin positive cells and their immunophenotype in HLA-DR2 positive and -DR2 negative subjects in NHS and PTB



Conclusion:

The present study suggests that HLA-DR1, especially HLA-DR1/DR2 combination is associated with higher macrophage phagocytosis. HLA-DR3 and -DR10 are associated with lower phagocytosis. DR2/DR3 combination is associated with lower phagocytosis in normal subjects. Moreover, HLA-DR2 positive pulmonary TB patients showed decreased perforin positive cells. This suggests that HLA-DR2 influences the innate immunity to *M. tuberculosis* infection.

Human Leucocyte Antigen (HLA) and non-HLA gene polymorphism studies in HIV and HIV-TB patients (ICMR Task Force project, funded by ICMR, New Delhi).

Background:

In developing nations, HIV-1 infection has increased the burden of TB, especially in populations where the prevalence of the infection is high among young adults. The importance of host genetic factors (HLA and non-HLA) on susceptibility or resistance and the variability of disease progression to HIV-1 infection have been emphasized by many studies.

Aim:

To find out whether HLA genes, HLA haplotypes and non-HLA genes are associated with the susceptibility or resistance to HIV and HIV-TB.



Methods:

The study subjects comprised of HIV negative TB negative (HIV-TB-) (n=150), HIV negative TB positive (HIV-TB+) (n=150), HIV positive TB negative (HIV+TB-) (n=150), and HIV positive TB positive (HIV+TB+) (n=150) groups. HLA-A, -B antigen were serologically determined. HLA-DR and -DQ and various non-HLA gene polymorphism were studied by PCR based DNA typing.

Results:

A trend towards a decreased antigen frequency of HLA-A11 is observed in HIV and HIV-TB patients. Moreover, an increased antigen frequency of HLA-B40, -DR2 was seen in HIV and HIV-TB patients than the control subjects. The above trends are based on 100 subjects in each group.

Performance of the Lysogenic phage Che12 LRP Construct phAETRC16 in sputum samples

Background:

Luciferase Reporter Phage (LRP) construct (phAETRC16) from Che12 was developed for increasing the sensitivity of the rapid diagnostic LRP assay.

Aim:

Evaluation of phAETRC16 for the diagnosis of TB

- a. In comparison with phAE129 using spiked sputum samples.
- b. On 0 day and 7th day using conventionally decontaminated sputum deposits treated with phagebiotics.

Method:

Smear negative sputum samples were spiked with *M. tuberculosis* suspension.

Readings were taken the same day, viz. six hours post infection with LRPs.

Sputum deposits obtained after processing by Petroff's method were suspended in 7H9 medium containing phagebiotics. Phagebiotics is the cocktail of three phages capable of controlling normal flora that survive the action of four per cent NaOH after Petroff's decontamination. About 122 smear positive sputum samples were tested – out of which 10 sputum samples gave RLU more than 500. These were considered as positive for LRP and analyzed.

Results:

Out of 30 spiked sputum samples tested, 12 gave RLU more than the cut off value of 500. The \log_{10} values are plotted in the graph (Fig.4) of the 12 samples, 9 showed higher readings with phAE129, and the remaining 3 showed no significant difference.

Statistical analysis using Wilcoxon Signed Ranks Test at 95 per cent significance level showed that there was a statistically significant difference between the spiked sputum readings of phAE129 and phAETRC16 readings ($p < 0.01$). The light out put of phAETRC16 on zero day and seventh day was compared and the readings of the 10 sputum samples were plotted as \log_{10} in the graph (Fig.5). Comparison of results showed only a marginal increase on the seventh day. The statistical analysis showed that there was no significant difference.



Conclusion:

- a) phAE129 was found to perform better than phAETRC16 in spiked sputum samples.
- b) A marginal increase in light output was observed on the seventh day in comparison with the zero day as expected with the lysogenic phage construct.

Fig.4: Log₁₀ values of RLUs of phAETRC16 vs phAE129 in spiked sputum samples

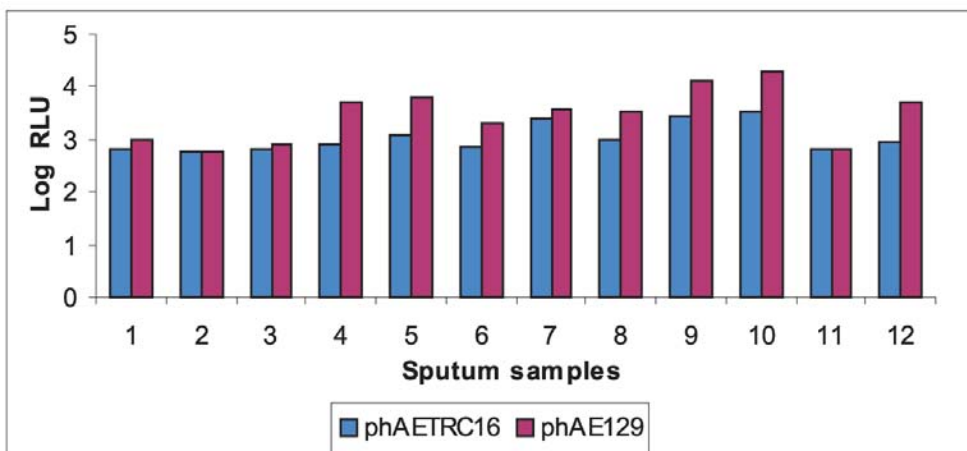
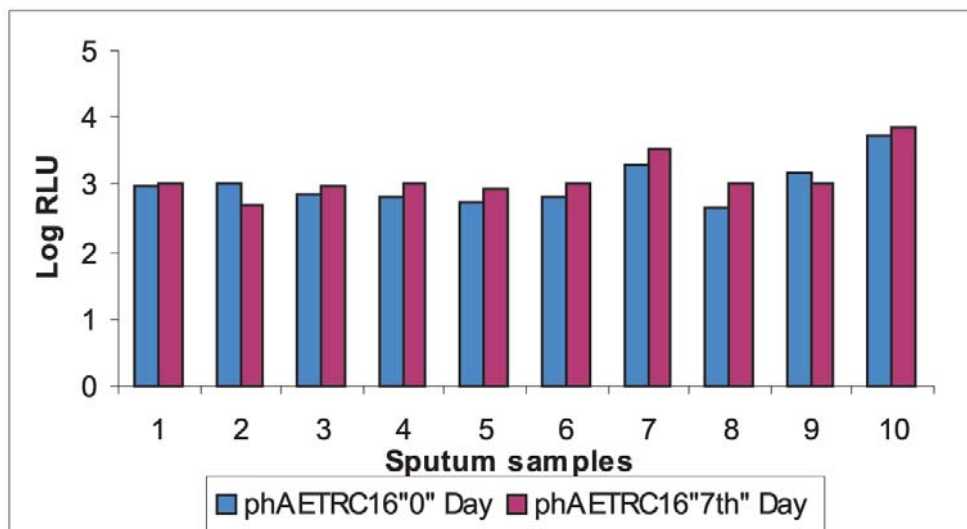


Fig.5: Log₁₀ values of RLUs of phAETRC16 on 0 day and 7th day in Sputum samples



Studies in progress:

Over expression of MSMEG3682', a protein kinase from *M. smegmatis* associated with its morphological changes

Background:

The genome of *M. smegmatis* (sequencing at TIGR), has 18 putative serine threonine kinases. While the genome of *M. tuberculosis*, contains 11 putative serine threonine kinases. MSMEG3682' of *M. smegmatis*, was the only protein homologous to both PknF and PknI of *M. tuberculosis*. Both of which are involved in cell division



in *M. tuberculosis*. We have reported for the first time a functional kinase from *M. smegmatis* and revealed its role in cell division.

Aims and Objectives:

To express the MSMEG3682 protein in *M. smegmatis* mc²155 under the control of acetamidase promoter, and study the effect of induction on growth, viability and cell morphology.

Methodology:

Standard molecular biology techniques were followed for the DNA manipulations and merodiploid strains constructed. For the induction experiments, 0.2 per cent acetamide (final concentration), was added to LB broth or solid media containing 0.05 per cent Tween 80. Growth was assessed by measuring OD₆₀₀ and viability by measuring CFU's. Cells were visualized by scanning electron microscopy. A total of 100 cells of each of the strains were measured for their length using the ANALYSIS software. These were then classified based on their sizes (in μm) into groups.

Results:

MSMEG3682 was over expressed in *M. smegmatis* mc²155, under the control of an inducible acetamidase promoter. Its effect on growth and viability was also studied. Changes in cell morphology were analyzed by scanning electron microscopy. Growth retardation was observed with the induced and uninduced merodiploid strain, compared to the wild type strain by three and 1.1 fold reduction (Fig.6). A corresponding difference in viability of three log and 1.7 log was found when plated on acetamide containing - LB plates (Fig.7). There was extensive clumping and cell lysis when merodiploid strain was grown beyond 30 hours. Scanning electron microscopy revealed irregular cell structure marked with bulb-like protrusions along the length of the cell or at the end of the induced merodiploid strain (Fig.8). Control strains failed to show differences in growth, viability and cell morphology.

Fig.6: Growth kinetics of merodiploid strain over expressing MSMEG 3682'

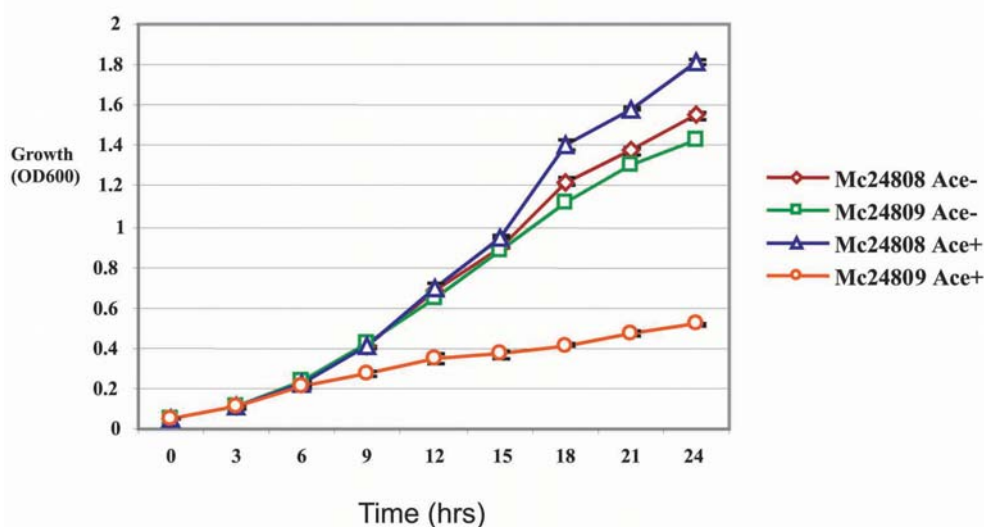
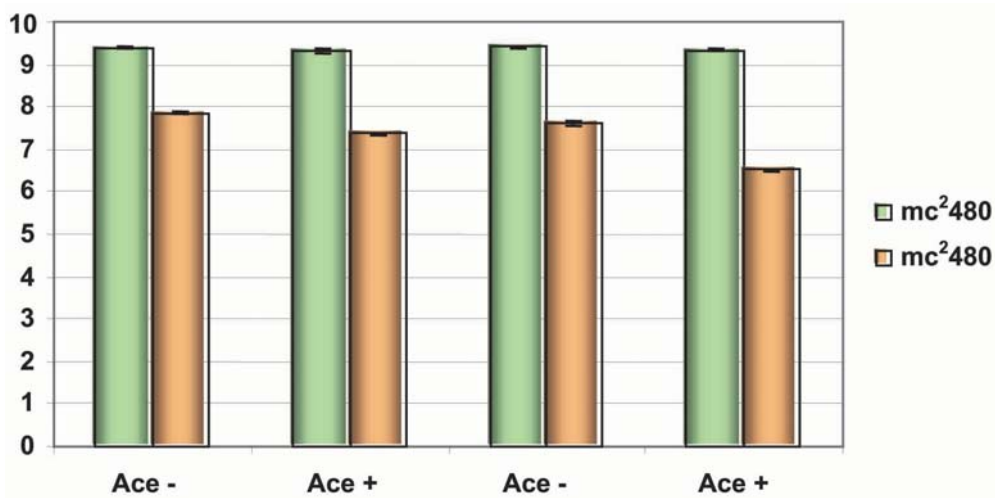




Fig.7: Viability of merodiploid strain over expressing MSMEG 3682'



Growth conditions in liquid and solid media

Fig. 8: Over expression of MSMEG3682' and gross morphological changes by SEM

Mc24808 uninduced



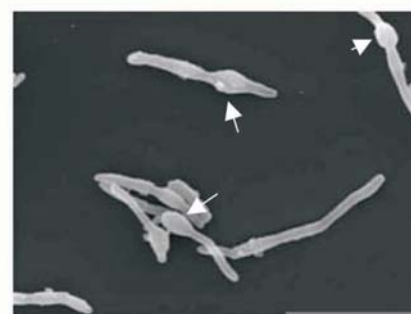
Mc24808 induced



Mc24809 uninduced



Mc24809 induced



Scale bar represents 5 μ m

Conclusion:

The study findings confirm our hypothesis that MSMEG3682' participates in controlling cell division and cell structure.

Protein Kinase E of *M.tuberculosis*: Implications in Nitric oxide Stress and Redox Regulation

Aims:

1. To create knock out mutants of *pknE*, *pknF* and *pknL* in *M.bovis* BCG by specialized transduction.
2. To study and see whether these genes are essential for the intra cellular survival of *M. bovis* BCG in THP1 macrophage cell line.
3. To study the role of *pknE* in stress response.

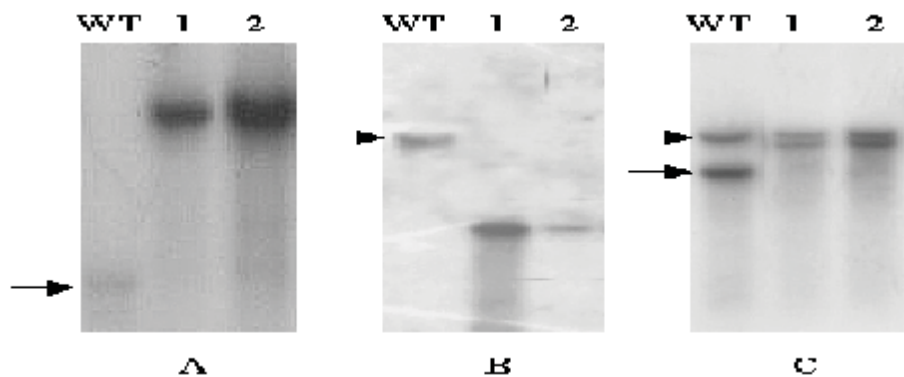
Methods:

Construction of gene disrupted strains of STPKs in *M.bovis* BCG and *M. tuberculosis*:

The *pknE* gene disrupted strains were generated by specialized transduction in *M. tuberculosis* and *M.bovis*. Briefly, the N-terminal and C-terminal flanking regions corresponding to 813 bp and 719 bp of *pknE*, were PCR amplified from the genome of *M. tuberculosis* H37Rv. These PCR products were cloned into pCR 2.1 (Invitrogen, USA), sequenced and subcloned into pJSC347. The SpeI and XhoI site in the first multiple cloning sites and the XbaI and StuI in the second multiple cloning sites, were used to insert the N-terminal and C-terminal flanking regions respectively. The Allelic Exchange Substrate (AES) was then cloned into the shuttle phasmid, pAE159 by *in vitro* packaging in *E. coli* resulting in ph159-PKNE. The recombinant phage DNA was electroporated into *M. smegmatis*, and high titres of the knock out phage was used to transduce *M. bovis* BCG-Pasteur in different bacilli-to-phage ratios. A genomic DNA was extracted and southern blot analysis of MluI digested DNA of both the strains was performed, using the N-terminal flanking region as the probe (result for the *pknE* mutant in *M. bovis* BCG is shown in Fig.9a).

A Δ *pknE* mutant was also constructed in *M. tuberculosis*, H37Rv. Similarly *pknF* and *pknL* disrupted strains were constructed in *M. bovis*. (Fig.9 b & b).

Figs. 9 a, b & c: Disruption of *pknE*, *pknF* and *pknL* in *M.bovis* BCG-Pasteur. Bands in the wild type lanes are indicated by black arrows (→). Southern Blot analysis of (A) the Δ *pknE* mutants (1 and 2) WT-4.1 kb, mutant-12.54 kb (B) the Δ *pknF* mutants (1 and 2) WT-7.524 kb, mutant-1.481 kb (C) the Δ *pknL* Mutants (1 and 2) WT-4.893 kb/9 kb, mutant-7.429 kb/9 kb.





Results:

PknE, *pknF* and *pknL* are non essential genes in *M.bovis* BCG-Pasteur and the corresponding mutants show different growth profiles *in vitro* and in a macrophage model of infection. *In vitro* growth kinetics showed that the *pknE* and *pknL* mutants were unaltered in their growth characteristics, when compared to wild type BCG. On the other hand, the *pknF* gene disrupted strain exhibited lower OD₆₀₀ values, indicating that it was impaired in *in vitro* propagated cultures (Fig.10).

The human macrophage model of infection, namely THP-1, was used to assess the growth and multiplication of the mutant. Although no significant differences were seen with the *pknE* mutant when compared to the wild type strain, both the *pknF* and *pknL* gene disrupted strains showed reduced rates of growth and multiplication within macrophages (Fig.11).

Fig.10: Growth kinetics of the $\Delta pknE$, $\Delta pknE::egfp$, $\Delta pknF$, $\Delta pknL$ (BCG mutants) compared to Wild type *M.bovis* BCG-Pasteur

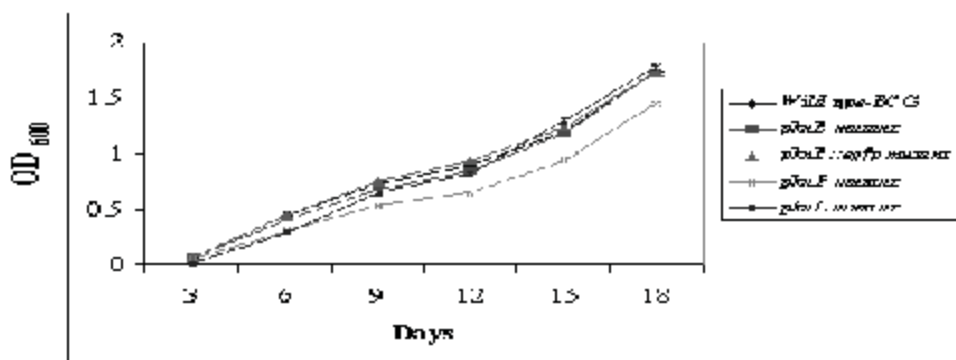
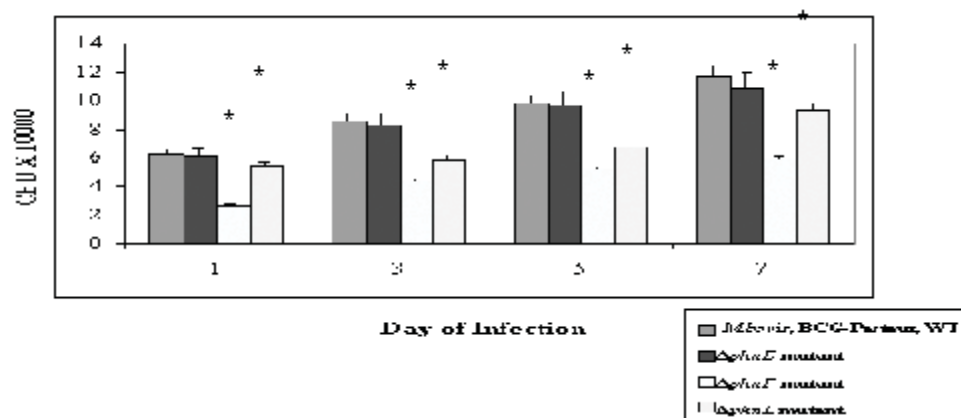


Fig.11: Macrophage survival assays for the $\Delta pknE$, $\Delta pknF$ and $\Delta pknL$ mutants of *M.bovis* BCG-Pasteur in a THP-1 model of infection. Colony forming units estimated at specified time points.

* $p < 0.05$.



The $\Delta pknE$ mutant of *M. tuberculosis* was tested against a variety of chemical compounds to determine whether there were any differences when compared to the wild type or the complemented strains.



The $\Delta pknE$ mutant was sensitive to hydrogen peroxide when compared to the wild type strain. But this difference was not statistically significant. On the other hand, the mutant was resistant to varying degrees to nitric oxide donors added to *in vitro* grown cultures of the wild type, mutant and complemented strains.

Molecular Epidemiology of *M. tuberculosis*

Background:

The amplitude of the TB problem world-wide and the global traffic requires the application of effective approaches to decipher the portable genotype patterns of this region, in comparison with the global patterns of disease transmission. The genetic information resulting from high prevalence areas such as ours would prove useful for defining phylogenetic links that exist with TB genomes, and also for constructing models of genome evolution. This will also help elucidate the evolutionary history of ancient tubercle bacilli.

Spoligotyping which interrogates the Direct Repeat (DR) locus of *M. tuberculosis* has the potential to identify the global distribution of the major clades of the *M. tuberculosis* complex. Spoligotyping is a PCR-based method dependent on hybridisation patterns of *in vitro*-amplified DNA with multiple spacer nucleotides. This region contains multiple short 36-bp direct repeats (DRs) and non-repetitive spacers, which are 36 to 41 bp in length, interspersed between the DRs. Spoligotyping is a rapid method that allows large numbers of isolates to be handled in a short time. The DRs are extremely well conserved among *M. tuberculosis* complex strains, making spoligotyping a specific method of detection of *M. tuberculosis* complex members.

Aims:

1. To spoligotype 1200 *M. tuberculosis* isolates.
2. To compare the genotype of the *M. tuberculosis* isolates from south India with that of the genotype of *M. tuberculosis* isolates from other parts of the world using the global spoligo database.
3. To identify the lineage of *M. tuberculosis* isolates from the Tiruvallur area.

Materials and Methods:

A standardized international protocol was used for spoligotyping. This is in accordance with the protocol supplied by the Isogen Bioscience BV, Manarssen, and instructions of the manufacturer. All spoligotype patterns were coded using the octal code system. These were then referred to a standardized international database of spoligotype patterns, SpolDB3 available at www.pasteur-guadeloupe.fr/tb/spol3. This was to determine whether each pattern had been previously reported.

Results:

Hundred different genotype clusters were identified with at least two different patients each who had the same spoligotype pattern. A total of 1038 (85.4 per cent) TB patients had a clustered isolate and 177 (14.6), had isolate with a unique spoligotype pattern. There was a median of three patients per cluster (range 338 patients). The spoligo patterns of all the isolates were compared to Spol DB₃,

Table 12: Classification of spoligotype patterns into major phylogenetic lineages



Major phylogenetic lineage	Number of spoligotype patterns	Percentage of spoligotype patterns	Number of Isolates	Percentage of Isolates
Beijing (I)	1	(0.4)	20	(1.7)
Beijing variants (I)	9	(3.3)	12	(1.0)
Delhi prototype (III)	3	(1.1)	26	(2.1)
Delhi variants (III)	20	(7.2)	28	(2.3)
Manila prototype (IV EAI)	1	(0.4)	1	(0.1)
Manila variants (IV EAI)	194	(70.0)	1034	(85.1)
Δ pks 15/1 (II)	41	(14.8)	83	(6.8)
Unknown	8	(2.9)	11	(0.9)
Total	277	(100.0)	1215	(100.0)

Groups (I, II, III, IV) Baker L. *et al.* 2004

The distribution of different spoligotype patterns into major phylogenetic groups is shown in Table 12. Most (70.0 per cent) of the spoligotype patterns were in Group IV, East African Indian, PGG1 (1), followed by the isolates in the Euro-American lineage (14.8 per cent) and the Delhi genogroup variants (7.2 per cent). Beijing lineage accounted for 20 prototype and 12 strains of Beijing variants, comprising about 2 per cent of the total isolates. Even more strikingly, 74.0 per cent of the clusters and 88.5 per cent of the clustered TB patients, had a spoligotype pattern in Group IV, followed by 14 per cent of the clusters and 5.4 per cent of the clustered TB patients in the Euro-American lineage.

Among the EAI group consisting of 1034 isolates, 30 per cent were of EAI class three, with the spoligo pattern of all spacers present, except 2-3, 29-32, 34, 37-39 spacers. Four per cent of the isolates belonged to EAI class 1 with all other spacers present, except spacer nos. 29-32, 34, and 40. The rest of the EAI isolates were variants of these 2 classes, yet to be defined.

Conclusion:

Our results showed that the major clade present in Tiruvallur belonged to the Group IV of Baker's classification that is named East-African Indian Lineage and close to the spoligo pattern of Manila isolates with the deletion at RD239.

Identification of immunoreactive T-cell antigens of *M. tuberculosis* through proteomic techniques

Background:

Even though effective chemotherapy is available for treatment of TB, there are practical difficulties in ensuring the desired high cure rate. This is due to many factors. Immuno-prophylactic measures using vaccines, is an alternative approach for control. A limited number of attempts to screen human responses to separated antigens have demonstrated that there are still numerous uncharacterized antigens



of various molecular masses to be evaluated. Moreover, a systematic approach to test the antigens purified by two-dimensional (2-D) preparative separations in human subjects, has not been attempted so far.

Aim:

To identify a set of immunologically relevant T-cell antigens and evaluate the response to these antigens in patients with TB and controls.

Methods:

The study subjects are as follows:

1. Apparently healthy household contacts (HHC) selected from families where there is at least one case of sputum positive pulmonary TB living in the same household. TB was ruled out in this group during the time of blood collection and hence considered "Protected".
2. Newly diagnosed adult pulmonary TB cases in the age group of 18-50 yrs. They form the "susceptible" group.

The methods:

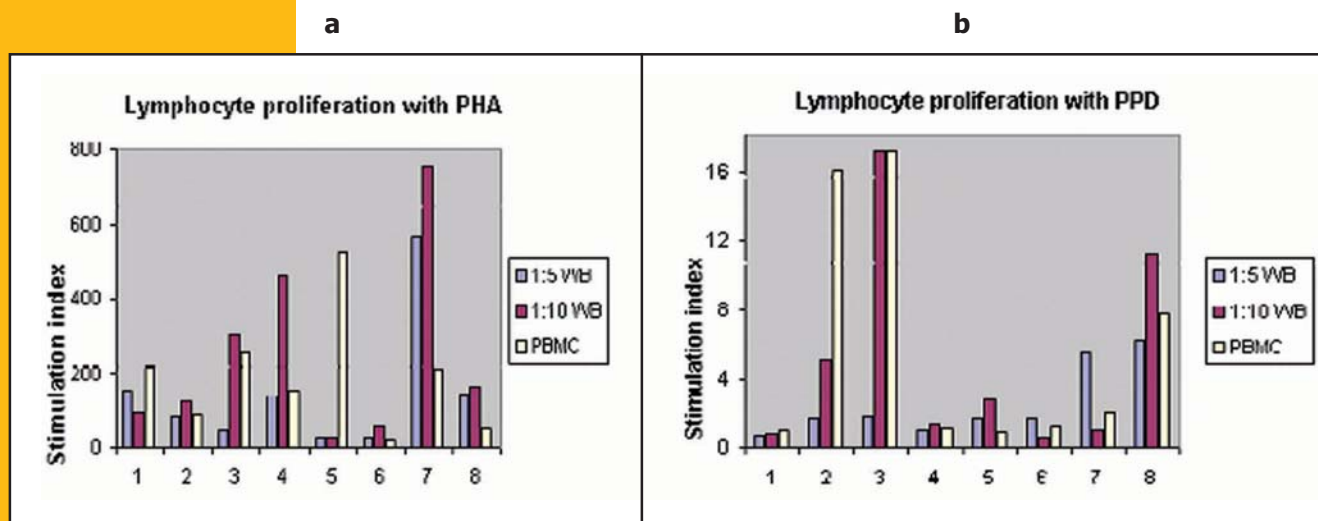
1. Two dimensional preparatory separation of antigenic fractions.
2. Proliferative response and IFN- γ response were studied using purified antigenic fractions.

Results:

Using the preparatory two -dimensional approach, the *M. tuberculosis* secreted proteins have been separated into 600 fractions. Of these, fractions having at least 50 μ g or more were selected for further testing (347 fractions). Since a large number of fractions will have to be tested in each blood sample, we have standardized a whole blood (1:10 dilution) assay, for proliferation and IFN- γ secretion (Fig. 13 a & b). It has been planned to compare at least 10 HHC and 10 TB subjects. So far five HHC and six TB samples have been tested.

Fraction showing differential stimulation and IFN- γ production, in the "protected" (HHC) and "susceptible" (TB) subjects, will be selected for further characterization.

Fig 13: Lymphocyte proliferative response in whole blood





Construction of luciferase reporter phages expressing Fflux gene driven by dormancy inducible conditional promoters

Background:

Early diagnosis of TB among the HIV-infected and general population is essential in reducing the morbidity and mortality. Over the past decade, LRPs have been developed. These show great promise in diagnostic microbiology. Conventional LRPs from lytic phages such as D29 and TM4 used in LRP assay, however highly specific, lack sensitivity. Hence it was hypothesized that temperate phage infecting *M. tuberculosis* if used in the construction of Reporter Phage System, would bring about a sustained light output. Moreover, most of the TB cases among the HIV-infected population result from the reactivation of latent bacilli and so LRP with a dormancy inducible promoter would identify viable, but not cultivable (VBNC) population of *M. tuberculosis*.

Aim:

To construct LRPs with dormancy inducible promoter driving Fflux gene.

Method:

Suitable cosmid vector with luciferase gene driven by hsp60 promoter was developed initially. Four efficient mycobacterial dormancy inducible promoters were identified and amplified from the H37Rv genome. A 368 bp PCR product of DevR promoter, 206 bp PCR product of Isocitrate-lyase promoter, 306bp PCR product of Nark2 promoter and a 220bp PCR product of Alpha Crystalline Protein (ACR) promoter, were cloned into the cosmid vector replacing hsp60 promoter individually. The presence of these conditional promoters was confirmed by sequencing the cosmid constructs. Using *Not1* site for cloning and lambda packaging, a functional LRP (phAETRC101) was developed from phAE159, a temperature sensitive mutant of TM4. Using the same strategy, similar constructs with dormancy inducible promoters from the temperate phage Che12, were developed.

Results:

LRP was successfully constructed using Che12 with Isocitrate-lyase promoter expressing Fflux gene (phAETRC21) and it gave a four-digit RLU reading with *M. smegmatis* mc²155 with integration time of 10 seconds. LRP construct with phAE159 having the alpha crystallin protein driving the luciferase gene was also developed (phAETRC102). The kinetics of phAETRC21 and phAETRC102 with *M. smegmatis* mc²155 was studied. Kinetics of phAETRC102 was studied both at 30°C and at 37°C to define the ideal temperature for sustained light output (Table 13). The RLU of phAETRC102 was greater than the Che12 based LRP construct phAETRC21 with *icl* promoter producing maximum light at 30°C (Fig. 14). Kinetics of these constructs was studied and compared with phAE129 (D29 based LRP) in a clinical isolate of *M. tuberculosis*. The RLU of phAETRC102 was greater than the rest of the two LRPs compared (Table 14 and Fig. 15).

Conclusion:

LRP constructs with dormancy promoters driving the *Fflux* gene show promise for use in rapid diagnostic assay for TB.



Table 13: Kinetics of LRPs at 30°C and 37°C in *M. smegmatis*

	30 Mins	3 Hours	6 Hours	24 Hours
phAETRC21	5730	31129	69825	10334
phAETRC102 at 30 degree C	118825	560000	644033	83766
phAETRC102 at 37 degree C	81570	156980	39293	560

Fig. 14: Kinetics of different LRP constructs in *M. smegmatis* mc2155

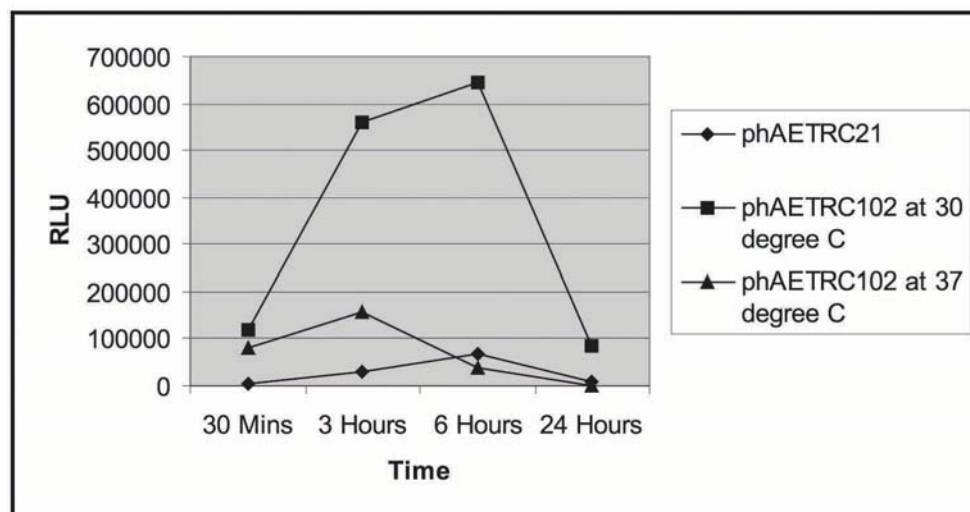
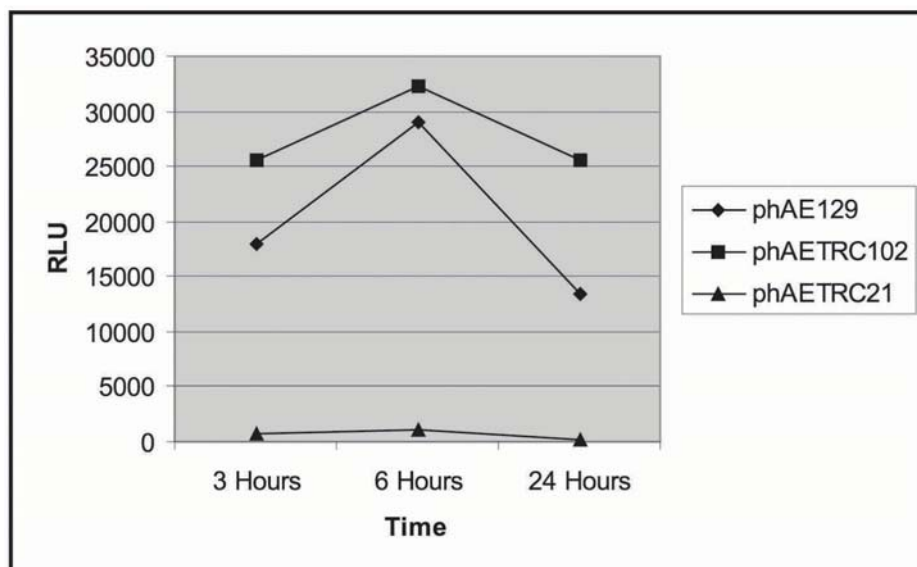


Table 14: Rapid diagnostic assay for tuberculosis

F9621	3 Hours	6 Hours	24 Hours
phAE129	17913	29046	13397
phAETRC102	25569	32271	25579
phAETRC21	698	1041	178

Fig.15: Kinetics of different LRP constructs in *M. tuberculosis* clinical Isolate No. RF 9721





An alternative method of sputum processing to detect *M. tuberculosis*

Background:

Conventional sputum processing by modified Petroff's method involves treating sputum specimens with 4 per cent NaOH, which is deleterious to tubercle bacilli to some extent. An alternate bio-friendly method that is mild on tubercle bacilli and at the same time harsh on normal flora exhibiting high mucolytic activity should result in increased detection of TB cases. In addition, it should help to improve the sensitivity of rapid diagnostic procedures as well. Chitin is present ubiquitously in the animal kingdom and is biofriendly. Its mucolytic activity and decontaminating ability could prove to be useful in developing an alternate sputum processing method.

Aim:

To develop a novel bio friendly sputum processing method, using chitin to detect tubercle bacilli.

Method:

A total of 120 samples were collected and divided into two parts. After randomization, one aliquot was processed with chitin-H₂SO₄ and another with modified Petroff's method as the gold standard. Both the deposits were inoculated onto LJ medium and randomized. Deposits were also inoculated onto blood agar medium to evaluate the effect of chitin on growth of normal flora.

Results:

Only 6 samples resulted in the growth of normal flora, surviving the action of chitin. Fifteen samples still showed the presence of normal flora when subcultured on blood agar (Fig.16). When smear results were compared with that of culture, Petroff's method performed better (kappa-0.77) as compared to Chitin method (kappa-0.67). Sensitivity of chitin processing method was 84 per cent and the specificity was 87 per cent. Culture results of chitin method showed an agreement of 85 per cent compared to the Petroff's method. The culture positivity of the smear negative samples was found to have statistical significance ($p < 0.001$) (Fig.17).

Conclusion:

Chitin has better mucolytic activity and decontaminating ability than 4 per cent NaOH. The *M. tuberculosis* retrieval capacity of both methods is the same. Hence Chitin can be an ideal alternative to NaOH for sputum processing.

Fig. 16: Growth of normal flora on blood agar medium

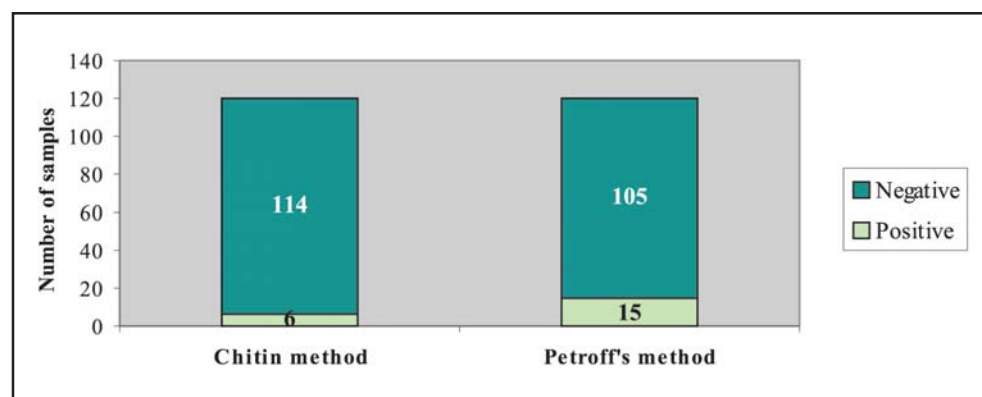
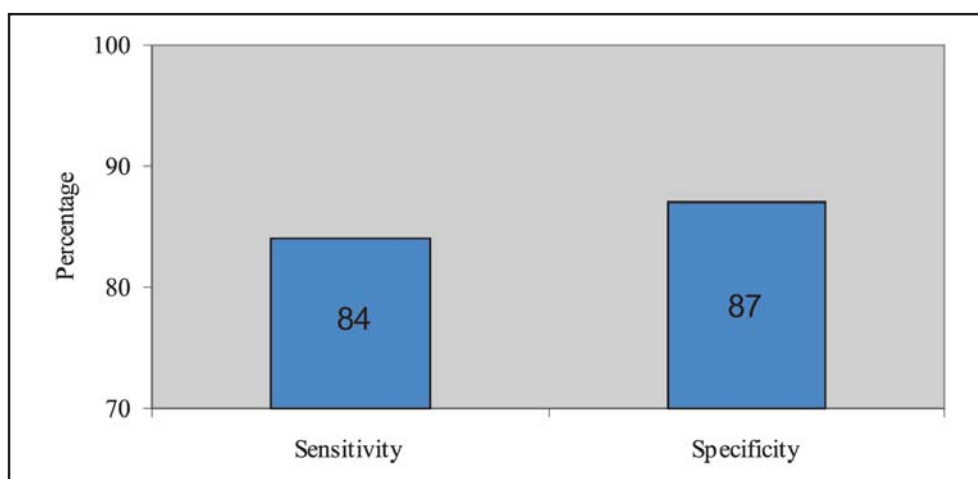




Fig.17: Sensitivity and specificity profile



Rapid screening of plant essential oils against *M. tuberculosis*

Background:

The resurgence of TB is one of the most serious public health challenges of the 21st century. The World Health Organization has given priority to the control of TB and prevention of the spread of drug resistant strains. There is an urgent need to discover novel anti-TB agents, especially from natural sources because of the increasing resistance of mycobacteria to the classic anti-TB drugs. The antimicrobial activity of plant oils and their extracts have been recognized for many years. Searching anti-TB compounds from plant essential oils, is a novel approach leading to the identification of new active molecules.

Luciferase Reporter Phage Assay is a rapid, inexpensive and less laborious method for high through screening of compounds against *M. tuberculosis*. This approach utilizes genetically engineered reporter phage to detect viable tubercle bacilli.

Aim:

To carry out Rapid Screening of plant essential oils for antimycobacterial activity by LRP assay.

Bioassay:

Nine essential oils (calamus, camphor, cinnamon, clove, eucalyptus, lemon, lemon grass, menthe, and Tulsi oil) were selected on the basis of traditional practices, literature, survey and laboratory evaluation against routine microbes to screen for antimycobacterial activity. The reference strain *M. tuberculosis* H37Rv, was used in the screening procedure. Two concentrations of the essential oils (100 and 50 μ g/ml) were used for screening by LRP assay.

Result:

Cinnamon oil showed 85.99 per cent activity against *M. tuberculosis*, followed by camphor with an activity of 69.72 per cent at 100 μ g/ml concentration (Table 15).

Conclusion:

Cinnamon oil is a promising candidate for anti TB drug development.

Table 15: Percentage RLU reduction of essential oils against *M. tuberculosis*



Oil name	% RLU Reduction	
	50 µg/ml	100 µg/ml
Calamus oil	6.27	41.02
Camphor oil	42.04	69.72
Cinnamon oil	65.44	85.99
Clove oil	36.81	40.22
Eucalyptus oil	39.79	43.62
Lemongrass oil	40.3	48.15
Lemon oil	44.15	48.25
Mentha oil	5.3	6.48
Tulsi oil	34.75	29.38

Immune response in tuberculous pleuritis: Expression profile of toll like receptors (TLR) on various immune cells and its role in tuberculous pleuritis (TP)

Background:

Toll Like Receptors are pattern recognition receptors that are key molecules for the orchestration of cross talk among many clinical manifestation of TB. It regulates both innate and adaptive immune responses. In TP, the protective immune response is generated by both innate and adaptive immune cells at the site of infection. In our previous annual report, a differential T-helper cell response was demonstrated by intracellular cytokine studies. In this report, we studied the role of TLR in tuberculous pleuritis.

Aim:

To determine the expression profiles of TLR2 and TLR4, on various cell subsets and its impact on the Th1/Th2 outcome.

Methods:

Peripheral Blood Mononuclear Cells (PBMC) and Pleural Fluid Mononuclear Cells (PFMC) separation was done by Ficol-Hypaque density gradient centrifugation. Pure population of CD4 T lymphocytes was obtained by MACS purification. Cell-subset profiling and TLR expression was performed by flow cytometry.

Results:

CD14+ monocytes expressed maximum TLR-2 and TLR-4 in both PBMC and PFMC. TLR-2 but not TLR-4 expression was upregulated on monocytes present at the site of infection (Fig.18 & 19). Expression of TLR-2 and TLR-4 was further enhanced in IFN-γ secreting T helper cells.



Conclusion:

TLR-2 on monocytes has a prominent role at the site of infection. Higher expression of TLR-2 in IFN- γ secreting T-helper cells, suggest that it is essential for optimal mycobacterial antigen-specific TH1 cell response.

Key message:

TLR-2 mediated adaptive immune response in tuberculous pleuritis.

Fig.18: TLR-2 expression on various cells of PFMC (N=7)

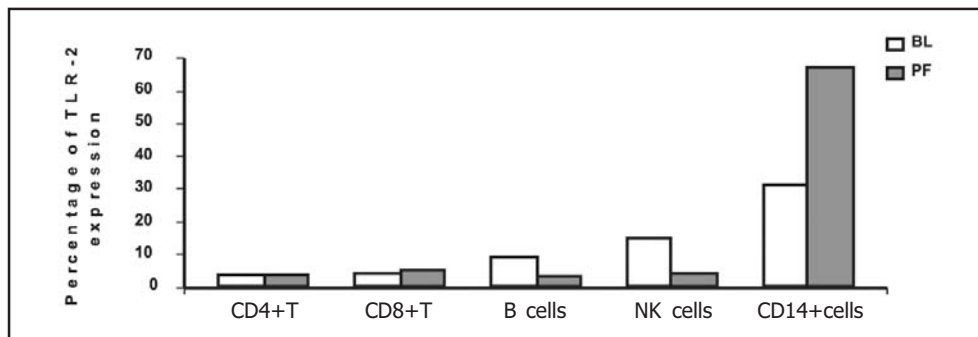
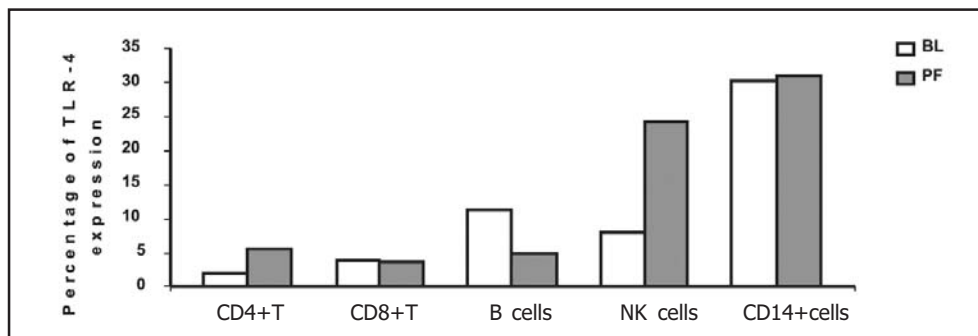


Fig. 19: TLR-4 expression on various cells of PFMC (N=7)



Molecular and Immunological characterization of *M. tuberculosis* strains with Single copy of IS6110: Modulation of immune markers and their correlation with apoptosis in THP-1 cells infected with prevalent strains of *M. tuberculosis*

Background:

The innate ability of infected macrophages to undergo programmed cell death (Apoptosis) and curtail the infection is crucial for the host defense. Although phagocytosis and intracellular killing mechanisms are highly effective in eliminating TB bacilli, some strains have evolved strategies to inhibit this microbicidal function and make use of macrophage for its successful and prolonged survival. Such modulation in macrophage functions by some *M. tuberculosis* strains is an emerging theme in pathogenesis.

Aim:

To study the induction of apoptosis in THP-1 cells infected with prevalent strains of *M. tuberculosis* and correlate with expression of CD14 and MHC Class-II molecules, TNF- α secretion, phagocytosis and colony forming units (CFU).



Materials and methods:

THP-1 cells were differentiated from the macrophages by PMA and subsequently infected with various strains of *M. tuberculosis* (H37Rv, H37Ra, prevalent and primitive clinical strains S7 & S10). The rate of apoptosis (Annexin-FITC), expression of CD14 and HLA-DR (FACS) and TNF- α (ELISA) were estimated.

Results:

The total percentage phagocytosis ranged from 60-80 in all the strains with no significance. However, based on phagocytic index (Fig.20), THP-1 cells infected with clinical strains showed low dose of infection in 1-10 bacilli category thereby exerted less burden on the cells. The uniform increase in CD14 expression was observed on all infected THP-1 cells, except H37Ra at all time points. Infection with clinical strain S7 induced less apoptosis (Fig.21) with significant decrease in HLA-DR, whereas S10 infected cells showed increased apoptosis with increase in CD14, HLA-DR and TNF- α levels at later time points.

Conclusion:

Our results indicated differential mode of infection by clinical strains which adopted the best strategy of “more spread but less burden” for their successful establishment in the host environment.

Key message:

Differential mode of infection by clinical strains and their adaptation to different survival strategies may lead to immune suppression and pathogenesis of the disease.

Fig.20: Phagocytosis of various *M. tuberculosis* strains by THP-1 cells

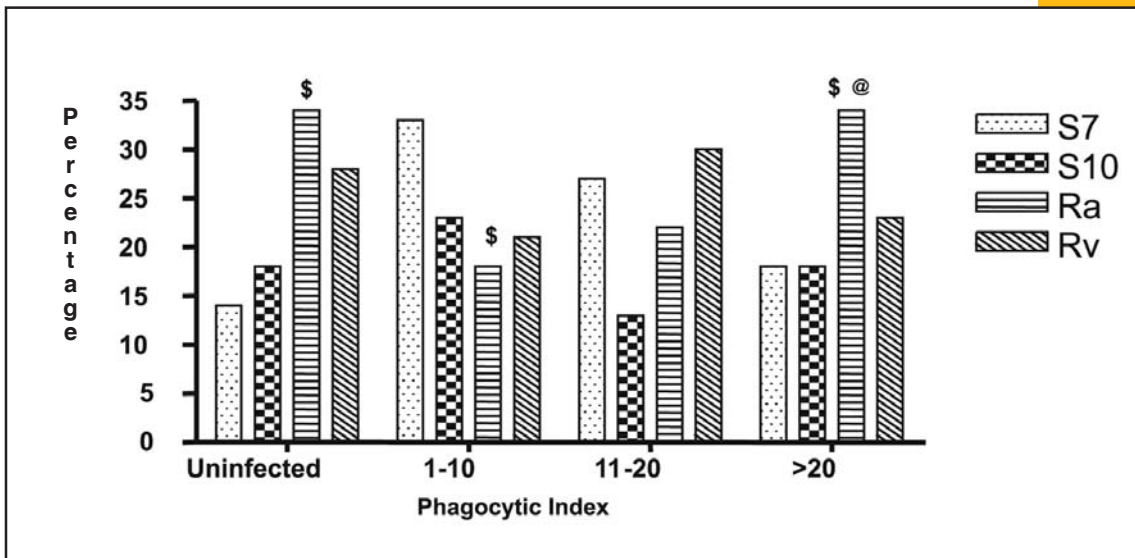
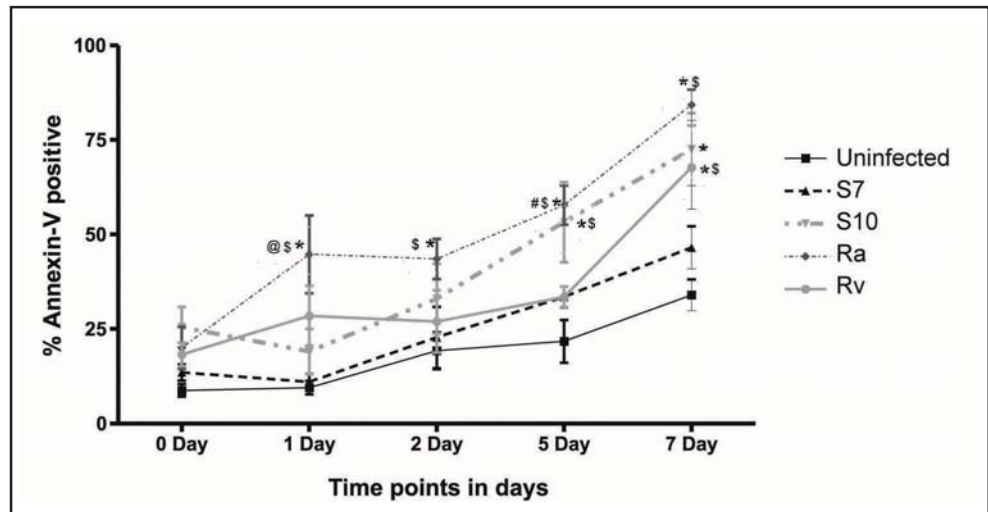




Fig. 21: Apoptosis induced by various *M. tuberculosis* strains



Role of chemokines in tuberculous Immunity: Expression profiles of chemokines and chemokine receptors on T cells in tuberculous pleurisy

Background:

Chemokines are known as the chemotactic cytokines that mediate the biological functions by binding to the specific G-protein coupled receptors exhibited on the surface of various immune cells and aid their transmigration to the site of infection. Cells of the innate immune system express various receptors for inflammatory chemokines, which is the first line of defense against the invading pathogen. Tuberculous Pleurisy is an effective model to study the role of chemokines at the site of active *M. tuberculosis* infection.

Aim:

- (i) To study the expression profile of chemokine receptors on T cells of blood and pleural fluid obtained from the tuberculous pleuritis patients.
- (ii) To quantify and compare the levels of chemokines and cytokines in the plasma and pleural fluid of the same patients.

Methods:

Pleural fluid and blood was collected from TB pleuritis patients.

The Pleural Fluid Cells (PF) and Blood Cells (BL) were labelled for various chemokine receptors and analyzed by Flow Cytometry.

Chemokines and cytokines were assayed by Cytometric Bead Array (CBA).

Results:

There was a significant increase in the expression levels of CCR2, CXCR2 and CXCR3 but not in CCR5 on CD4⁺ T cells of PF compared to BL.

Similar increase of CCR1 and CCR7 was observed on CD3⁺ T cells of PF (Fig.22 & 23). The dual function chemokines (MIG and IP-10) and inflammatory chemokines (MCP-1, IL-8 and MIP-1 α) were significantly elevated in PF compared to BL. RANTES was significantly high in blood (Fig.24 & 25).



Conclusion:

Tuberculous Pleurisy is characterized by lymphocyte predominance mounting protective Th1 response. A selective concentration of chemokines, cytokines and abundant expression of chemokine receptors further confirm the accumulation of activated and memory T cells at the site of infection and help in polarizing the immune response.

Key message:

Chemokines and chemokine receptors help in migration of activated immune cells to the site of infection.

Fig. 22: Expression of α and β chemokine receptors on CD4+ T cells

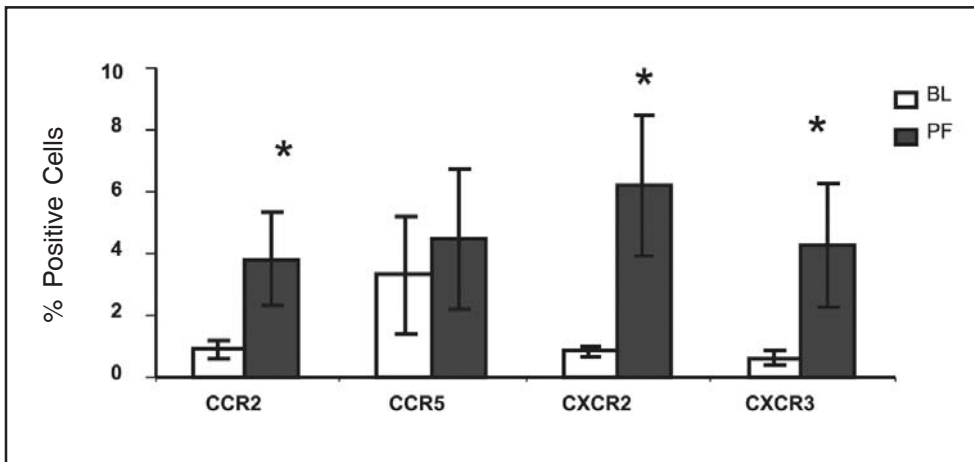


Fig. 23: Expression of β type of chemokine receptors on CD3+ T cells

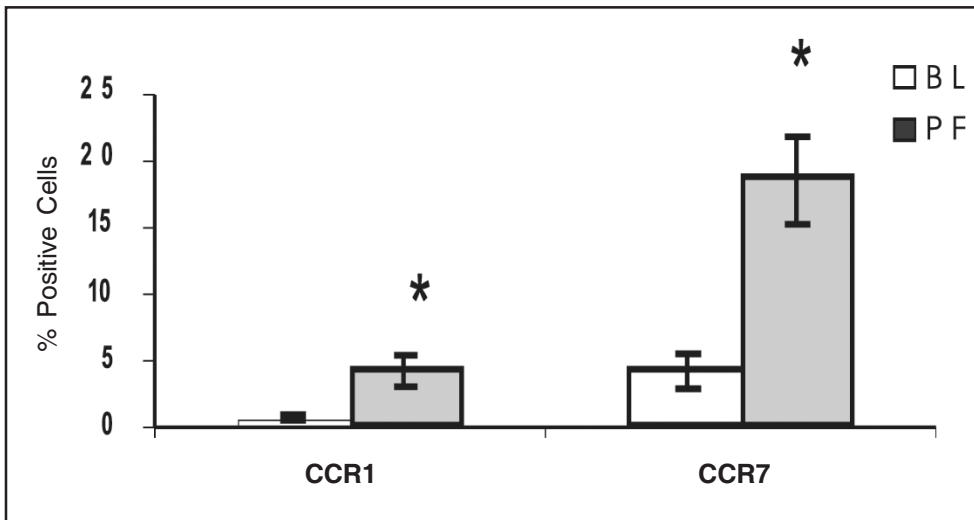




Fig. 24 : Levels of dual function chemokines in blood and pleural fluid

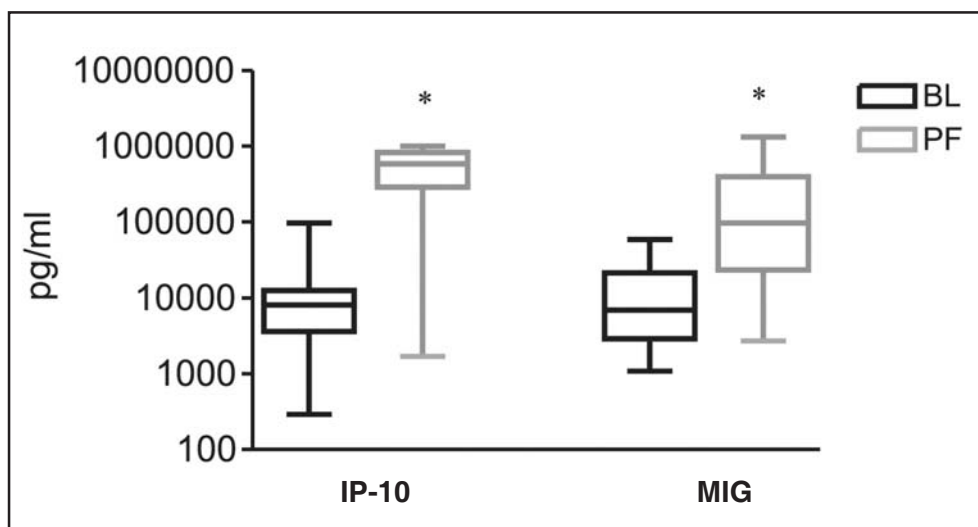
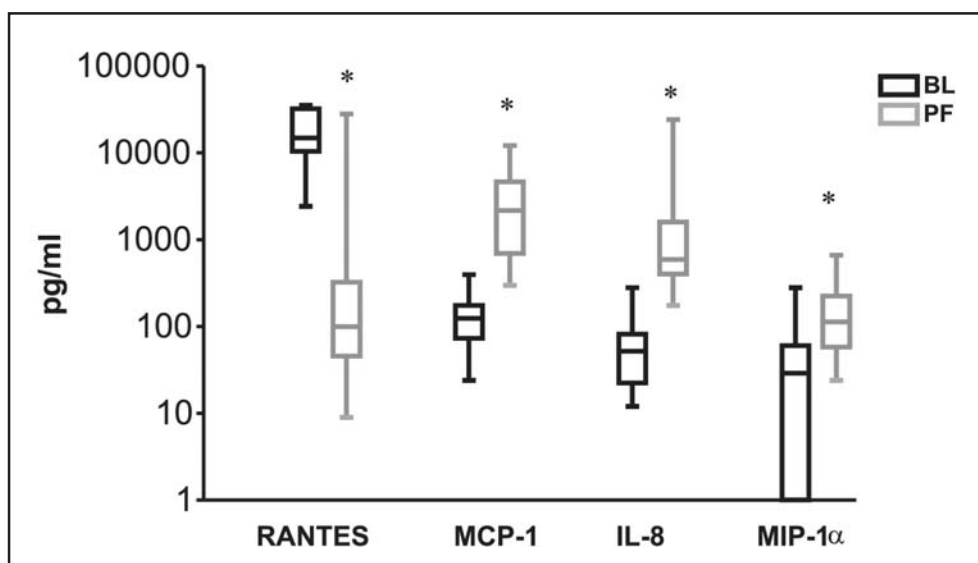


Fig. 25: Levels of inflammatory chemokines in blood and pleural fluid



Role of Dendritic cells (DC) in Mycobacterial Immunity: Differential maturation of human monocyte derived – dendritic cells by prevalent strains of *M. tuberculosis*

Background:

Dendritic cells play a key role in the pathogenesis of TB. The primary initiation and modulation of immune response is mediated through the DCs. Maturation of DC is necessary for effective priming of immune response.

M. tuberculosis has developed various mechanisms to evade host immunity. One such strategy is to inhibit the maturation of DC. It has been reported that laboratory virulent strains suppress DC maturation. Similarly the prevalent clinical strains may prove a better source for studying the association of virulence with evasion of immune mechanism by DCs.



Aim:

The study has been designed to evaluate changes in the functional and phenotypic maturation of monocyte-derived dendritic cells (MDDC) upon infection with prevalent strains (S7 and S10) and laboratory strain (H37RV) of *M. tuberculosis*.

Methods:

PBMCs were isolated from the blood of healthy volunteers. Monocytes were purified by using anti-CD14 conjugated magnetic beads. MDDC were generated by culturing CD14⁺ cells with GM-CSF and IL-4 for six days. On day seven, the MDDC were infected at a *Multiplicity of infection (MOI)* of four with various mycobacterial strains. After 24 hours, DCs were harvested and analyzed using FACS for various phenotypic markers. ELISA for cytokines and chemokines was performed in the culture supernatants.

Results:

Upregulation of CD80 but not CD86, was seen after infection with Rv & S7 whereas S10 showed significant increase in CD86 (Fig.26). There was increased CD83 expression after LPS stimulation and S10 infection but not in Rv and S7 infected DCs. Down regulation of CCR5 and upregulation of CCR7 was observed in all infected DCs. The levels of IL12p-40 and TNF- α also increased after infection with all the strains. There was marginal increase in IFN- γ levels with no change in IL-1 β after infection. The IP-10 levels were significantly increased in LPS and S10 whereas MIP-1 α levels (Fig.27) significantly decreased in Rv infected DCs.

Fig. 26: Expression of costimulatory and maturation markers on DC

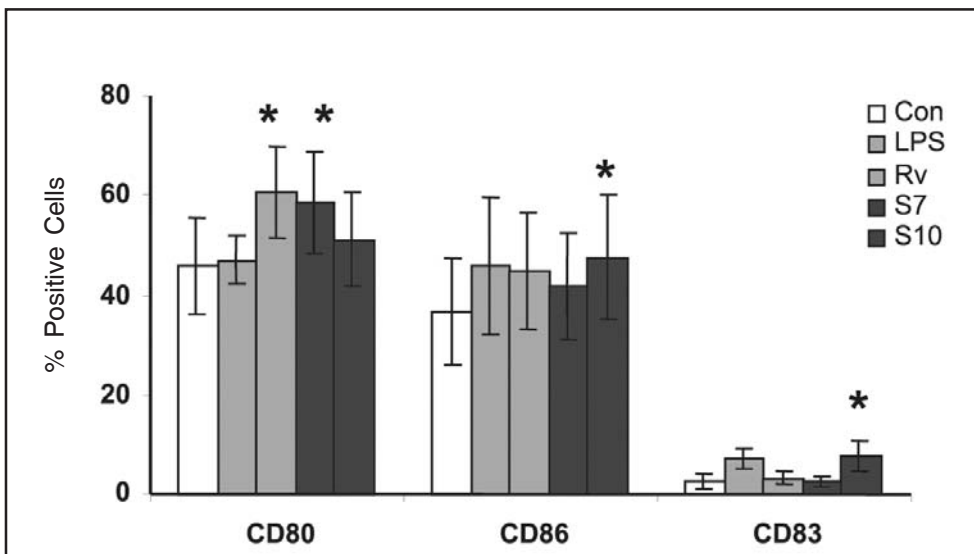
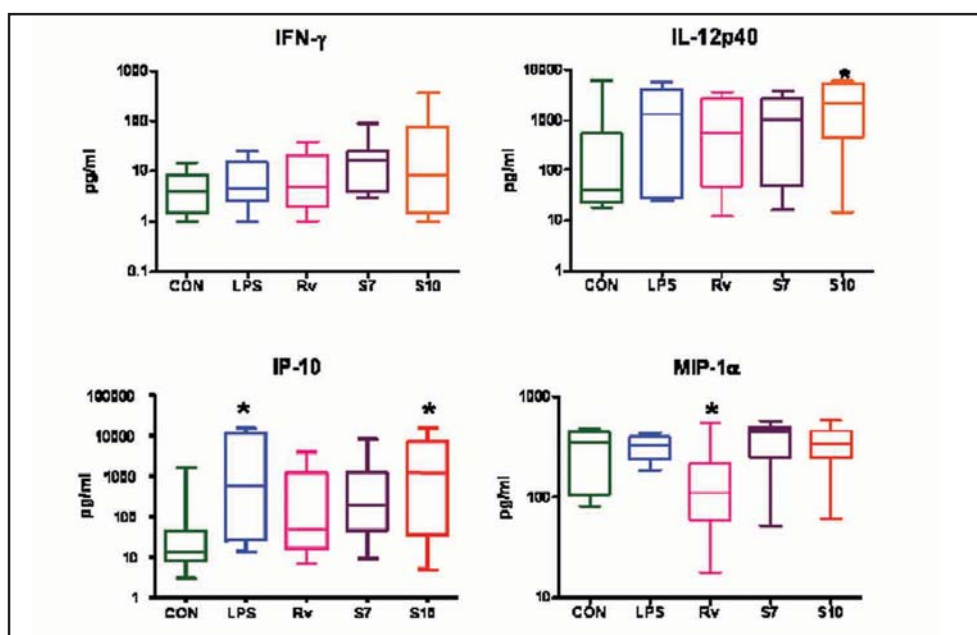




Fig. 27: Cytokine and chemokine profile of infected DCs



Conclusion:

The maturation and function of DCs are modulated by the *M. tuberculosis* strains depending upon their virulence. The laboratory strain H37Rv and clinical strain S7 are highly virulent compared to S10 in inhibiting the complete maturation of DCs. Thus the hampering of maturation of DCs is a novel mechanism adopted by *M. tuberculosis* strains to evade the effective immune response of the host.

Key message:

M. tuberculosis strains hamper DC maturation as a strategy to survive in the host.

The role of complement in the interaction of *M. tuberculosis* with human macrophages

Background:

The complement system, an important component of innate immunity is a potent mediator of inflammation and at the same time plays a pivotal role in modulating the adaptive immune response also. The initial interaction between the complement system, the macrophage and *M. tuberculosis* is an important first step in the pathogenesis of tuberculosis and is mediated by specific macrophage receptors and ligands present on the surface of *M. tuberculosis*. Although it is known that *M. tuberculosis* replicates within the host macrophages, the mechanism by which it evades being killed by macrophages remains poorly understood. Since mycobacteria interact with the complement system initially and as antimycobacterial antibodies are known to be present in endemic populations, it was considered important to investigate whether antibody could modulate complement activation and determine the interaction of *M. tuberculosis* with the macrophage.

Aim:

To study whether antibodies could modulate complement mediated interaction of *M. tuberculosis* with the macrophages.



Methods:

Phagocytosis of *M. tuberculosis* by macrophages was assessed under the following conditions:

1. Different infection ratios (macrophages:mycobacteria).
2. Different serum concentration.
3. Pre opsonization of *M. tuberculosis* with serum at different time points of incubation.

Complement receptor expressions will be assessed in PBMC using flow cytometry. The levels of different inflammatory cytokines will be measured using flow cytometry.

Results:

The percentage of macrophages infected with heat-killed *M. tuberculosis* was lower than those with live bacilli was independent of the infection ratio used (Fig. 28). Association of *M. tuberculosis* was markedly decreased in heat inactivated pooled human serum (HI-PHS) ($48.66 \pm 8.17\%$) or under serum free condition ($45.5 \pm 9.04\%$). The ability of macrophage phagocytic index increases with increasing serum concentrations (Fig. 29). Pre opsonization of the bacilli with fresh serum also resulted in an increase in the phagocytic index (Fig. 30).

Fig. 28: Effect of infection ratio on phagocytosis of *M. tuberculosis* by macrophages (Mean \pm SE)

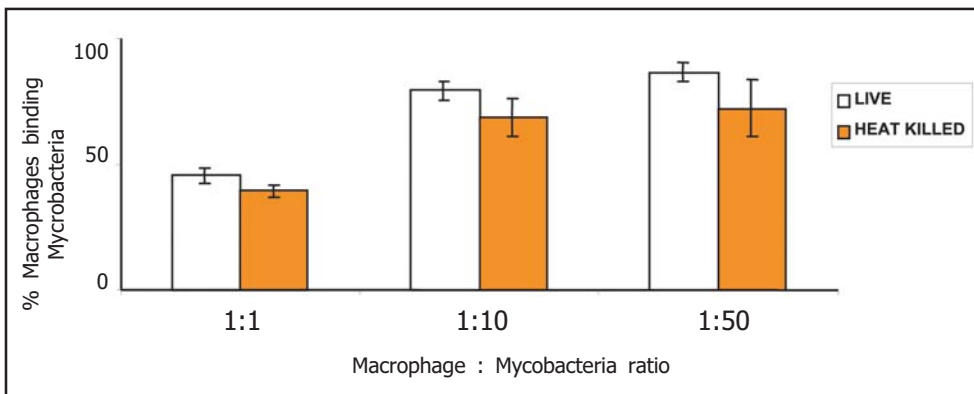


Fig. 29: Effect of opsonization (serum concentration) on phagocytosis of *M. tuberculosis* by macrophages (Mean \pm SE)

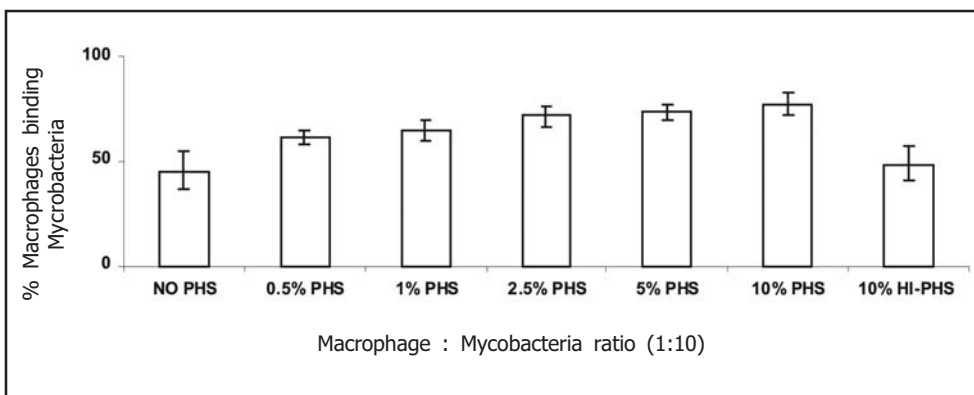
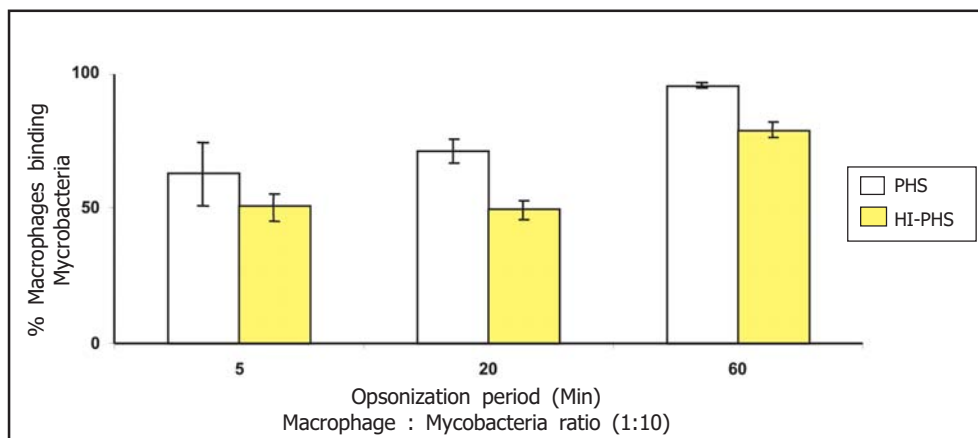




Fig. 30: Effect of opsonization (time) on phagocytosis of *M. tuberculosis* by macrophages (Mean \pm SE)



The status of the complement system in tuberculosis

Background:

The complement system serves as a major link between the innate and the adaptive immune systems as well as in facilitating production of appropriate immune response to an antigenic stimulus. The following are known about the involvement of the complement system in mycobacterial infections: Mycobacteria and some of their components like PGL of *M. leprae*, cord factor and PPD of *M. tuberculosis* activate the complement system. Complement components are found in circulating immune complexes formed in active tuberculosis. Levels of complement proteins and hemolytic complement are significantly higher in patients compared to healthy controls. Mice deficient in complement components are more susceptible to *M. tuberculosis* infection than control mice.

Aim:

To investigate the status of the complement system in tuberculosis by documenting the quantitative changes that occur in this disease, functional aspects of the complement system and the effect of complement components on the host immune responses in pulmonary tuberculosis.

Objectives:

Measurement of the levels of complement components and activation fragments in serum and complement receptors on peripheral blood lymphocytes.

Studying the alteration of the functional capacity of the activated complement system in tuberculosis.

Documenting the effect of complement on the immune responses against *M. tuberculosis*.

Participants:

The study subjects will comprise of 25 patients each with active, smear positive pulmonary tuberculosis and subjects who have completed the full anti-tuberculosis treatment regimen and 50 normal healthy volunteers.



Methods:

Complement profile in tuberculosis:

The levels of complement components and their activation fragments were measured in serum using sandwich ELISA and the surface expression of complement receptors on peripheral blood mononuclear cells using flow cytometry.

Functional assay:

Functional characterizations of complement activation were assessed using ELISA using coated IgM for the classical pathway and cell surface glycoproteins of mycobacteria for the alternative pathway.

Effect of the complement system on host immune response:

The functional significance of the activated complement system on important host immune responses against *M. tuberculosis* were studied by analyzing the levels and components of circulating immune complexes using ELISA, and the effect of added complement components on phagocytosis and apoptosis of mononuclear cells using flow cytometry.

Results:

Mannose Binding Lectin (MBL) levels were found to be higher in both active and healed tuberculosis groups showing the involvement of the lectin pathway in the activation of the complement system. Also, a positive correlation was observed between MBL and C3d levels which is suggestive of the role of lectin pathway in potentiating complement system activation.

The increase in the complement activation fragments like C3d, C3a, C4a and C5a in the before treatment group reflects the disturbances in the host immune response leading to the activation of the complement system. The levels of C3 in serum from pulmonary tuberculosis patients were found to be less compared to normal healthy volunteers. This may be due to excess activation of the complement system leading to cleavage of C3 to its activation fragments.

The levels of circulating immune complexes and the presence of complement components (C3, C3d, C4c) and immunoglobulins (IgG, IgM) in the complexes were found to be elevated in active tuberculosis group compared to controls. The data obtained from flow cytometry show significantly decreased expression of complement receptors in the major subsets of lymphocytes obtained from the before treatment group compared to the treated individuals and normal controls (Fig. 31 & 32). This points to the involvement of complement receptors in the immune responses of pulmonary tuberculosis and suggests that the expression levels are modulated by the underlying disease status of the patients.

Complement admixture was found to augment apoptosis since the percentage of cells undergoing apoptosis was more in mononuclear cells incubated with serum as source of complement compared with cells incubated with heat inactivated serum (Fig.33).



Fig. 31: Expression of CR1 on B lymphocytes

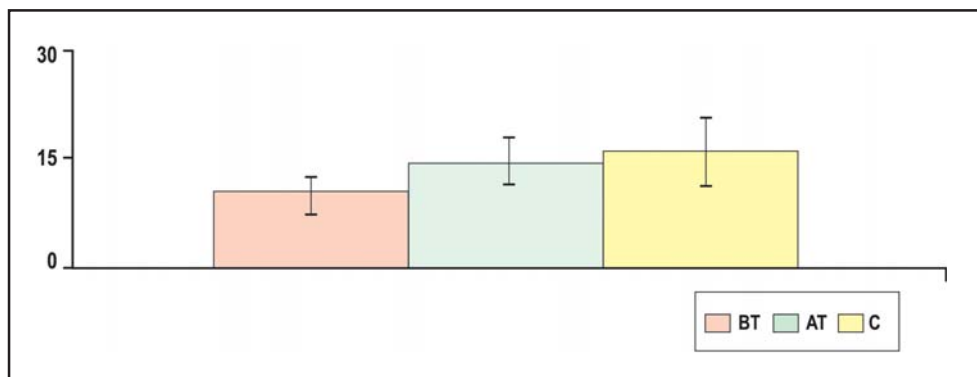


Fig. 32: Expression of CR2 on B lymphocytes

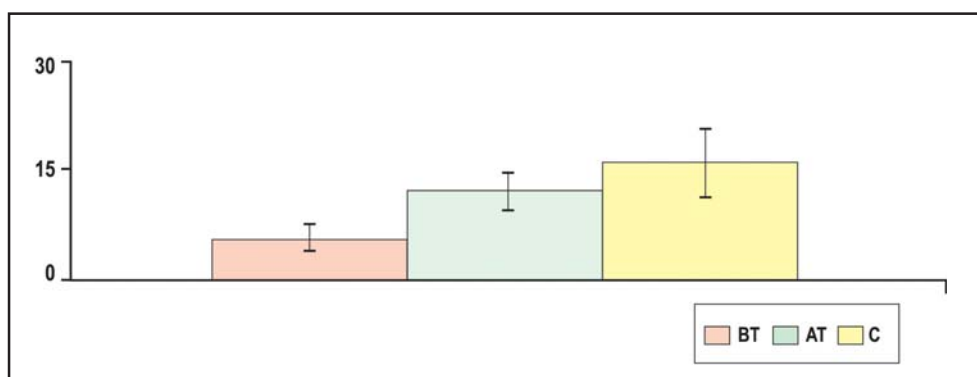
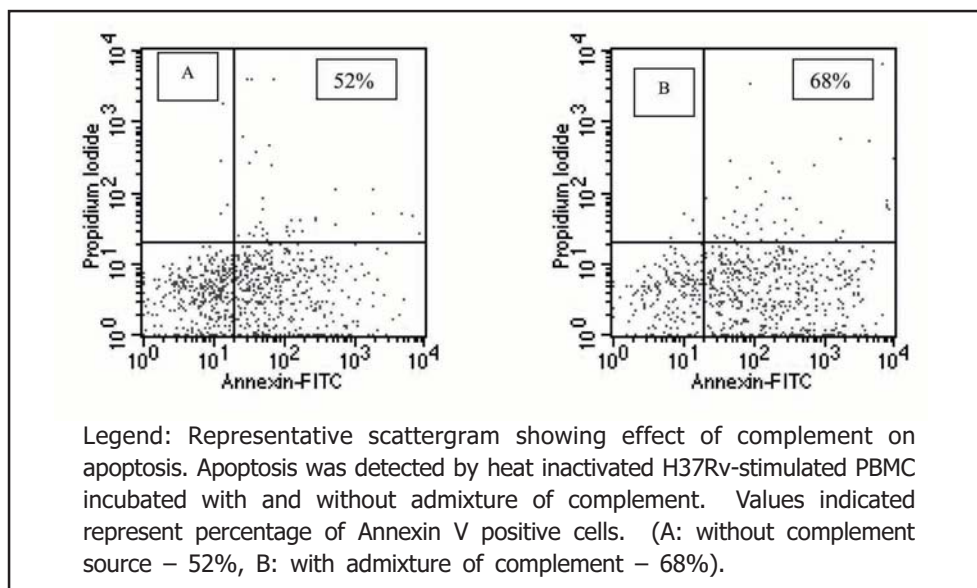


Fig.33: Augmentation of apoptosis by complement





Complement activation by gene-disrupted *M. tuberculosis*

Background

Complement system plays an important role in the opsonization and phagocytosis of mycobacteria. The alternative pathway of complement activation has the ability to recognize a variety of pathogens independent of antibody. However, both classical and alternative pathways are required for optimal phagocytosis of bacteria. Many studies have documented the complement activation potential of various mycobacterial strains and their cellular components. A number of gene-disrupted strains of *M.tuberculosis* are now available and differences in the pathogenic potential of these strains compared to the wild type strain are being delineated now. In view of the importance of the the innate immune responses in modulating the host-parasite interactions, it is important to investigate the relationship between the complement system and genetically modified strains of *M.tuberculosis*.

Objectives:

To assess complement activation at the level of C3, C4 and factor B by the following gene-disrupted *M. tuberculosis* strains: MtpA, MtpB, VirS, DKO, DevR, complemented strains of all these except DKO and their respective wild strains, *M. tuberculosis* Erdman and H37Rv strains.

To assess the effect of the above mentioned strains of *M.tuberculosis* to modulate the expression of complement receptors on peripheral blood leucocytes and release of various cytokines from them.

Methodology:

All the above strains are grown in Middlebrook 7H9 broth for 3-4 weeks at 37°C. Complement activation at the level of C3, C4 and factor B by *M.tuberculosis* will be assessed using solid phase ELISA.

Complement receptor expression will be evaluated using flow cytometry and the quantitation of the various cytokines (VNR to mention the cytokines) using ELISA.

Results:

The kinetics of the uptake of C3 and C4 by the bacilli has established that this is dependent on both the concentration of the bacilli and serum.

C3 and C4 uptake by the knock-out bacilli was found to be inversely related to number of bacilli.

Decreased C3 uptake through both pathways was observed for DKO and DevR strains compared to their respective wild strains, Erdman and H37Rv.

C4 uptake was found to be higher for all gene-disrupted strains compared to their wild strains; and inversely related to the number of organisms.

For the complemented strains, C3 uptake was found to be higher by MtpA complemented strain compared to the wild strain; VirS complemented and DevR complemented strains exhibited less values or almost equal to those of their respective wild strains (Fig. 34-36).

C4 uptake was found to be higher by MtpA complemented, VirS complemented, and DevR complemented strains, though not significant.



Fig. 34: Uptake of C3 by MptpA and the complemented strain compared to the wild type

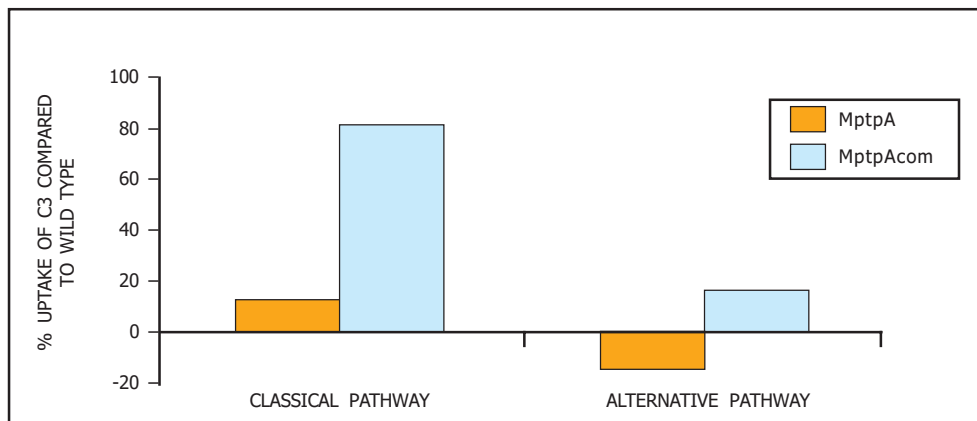


Fig. 35: Uptake of C3 by DevR and the complemented strain compared to the wild type

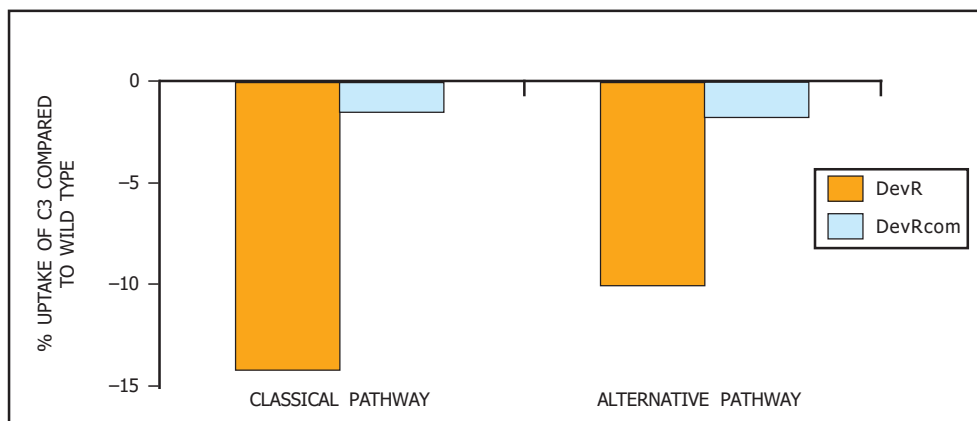


Fig. 36: Uptake of C3 by VirS and the complemented strain compared to the wild type

