

5. APPLIED RESEARCH

Completed Studies:

Pot-staining of sputum for detection of acid-fast bacilli

Background:

For over a century the diagnosis of pulmonary TB is being confirmed by detection of acid fast bacilli (AFB) in direct smears of sputum made on glass slides. Good laboratory practices have to be followed while making direct smears from the mucopurulent portion of sputum on glass slides to avoid laboratory acquired TB infection. Generally, making direct smears from sputum is considered hazardous by laboratory technicians working in developing countries with limited facilities. In some higher level laboratories, smears are made from deposits after processing sputum samples. These deposit smears, in addition to generating aerosols often get peeled off from the slides resulting in false-negative results. In Ziehl-Neelsen (ZN) method, the carbol-fuchsin solution poured on the slide is to be heated until vapour rises and should be allowed to remain that way for five minutes. In order to overcome these problems, there is a felt need for a new technique to stain the AFB, which is both simple and non-hazardous. Recently, it has been shown that the deposit of sputum sample, obtained after decontamination with 4% sodium hydroxide and concentrated by centrifugation, can be stained with 1% carbol-fuchsin in its container and its smears made subsequently on glass slide can be decolorised and counter-stained by the procedures followed in ZN method.

Aims:

- To stain the sputum samples in their containers and subsequently decolorise and counter-stain their smears (pot-smears) for detection of AFB
- To compare results of pot-smears with their corresponding direct-smears stained by the hot ZN method

Methods:

Seven hundred and twenty five direct smears made from sputum samples of pulmonary TB patients were stained by the hot ZN method for detection of AFB. Each of the above sputum sample was then mixed well in its container with 'phenol ammonium sulphate basic-fuchsin' solution (Fig.5.1) and its pot-smear, made after 1 hour (Fig.5.2), was then decolorised and counter-stained by the same procedures followed in ZN method (Fig.5.3). The coded direct and the corresponding pot smears were graded and the results compared.

Results:

Of the 725 samples, 212 and 213 were AFB positive by direct and flocculate-smears respectively and the difference observed in the smear results was not statistically significant.

Fig.5.1 : Pot-stained sputum samples

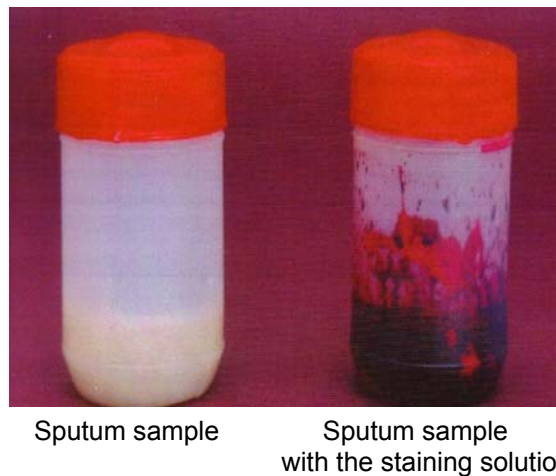
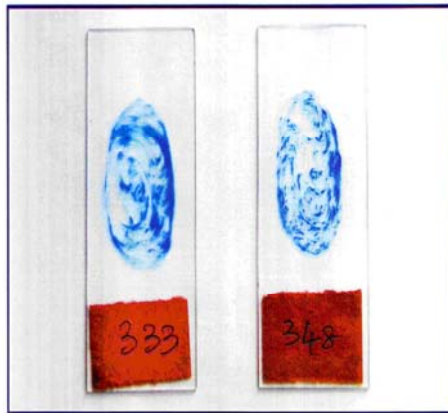


Fig.5.2: Smears made from pot-stained sputum samples



Fig.5.3: Counter stained pot-smears



Conclusion:

The sputum samples in their containers can be stained with 'phenol ammonium sulphate basic-fuchsin' solution and their smears made subsequently on glass slides can then be decolorised and counter-stained for detection of AFB. The results of pot-smears and direct smears are comparable.

(PI: Dr.N.Selvakumar; selvakumarn@trcchennai.org.)

Standardisation and evaluation of optimized diagnostic Luciferase reporter phage (LRP) Assay

Background:

Luciferase reporter phage assay (LRP) has the potential for use in diagnosis and drug susceptibility detection of *M. tuberculosis*. Though reported to have high specificity, LRP assay lacks sensitivity. An attempt was made to optimize the assay format to improve its sensitivity. One approach was to delay the premature lysis of the infected host cell through lysis inhibition. Subsequent infection of the host cells prior to cell lysis, results in continuous availability of cellular adenosine triphosphate (ATP) and increased copy number of the enzyme. A second approach was to increase the permeability of the cell membrane to facilitate transport of the substrate D-luciferin using dimethyl sulfoxide (DMSO).

Aim:

- To optimize the LRP assay format to improve its sensitivity

Methods:

Standardization experiments were set up with culture suspensions of *M. tuberculosis* H₃₇Rv, multi-drug resistant and Sm, H, R, E (SHRE) sensitive clinical isolates. Cells infected by lytic LRP construct (phAE129) and lysogenic construct (phAETRC16) were reinfected with phAE129 at different time points and the cell phage mixtures were incubated at 37°C. Control was setup with mycobacteriophage (MP) buffer instead of phAE129 at the time of reinfection. Photons released were measured at different time intervals as relative light units (RLU) in the luminometer.

D-Luciferin reconstituted in DMSO and made up in sodium citrate buffer was compared with that reconstituted in distilled water and made up in sodium citrate buffer. The cell phage suspension was prepared as per standard protocol for LRP assay using *M. smegmatis* mc²155 and phAE129.

Using the above modifications in the assay format, LRP assay was done in sputum deposits of 67 samples processed by modified Petroff's method. About 300 µl of deposit was added to 3 ml of modified 12B medium supplemented with antibiotics PANTA and incubated at 37°C. A 1/10 dilution of the deposit and LJ slopes were inoculated. On days 3, 6, 9 and 12, LRP assay was set up in the optimized format and readings were taken at different time points. LJ slopes were read every week till eight weeks.

Results:

Lysis inhibition experiments revealed that second infection of the cells by phage produced marked increase in light output when compared to the control. D-Luciferin in 2.5% DMSO gave one log higher RLU than D-luciferin by standard preparation.

Among the 67 samples tested, conventional LJ culture was positive for 25 samples and negative for 42 samples. LRP assay was positive for 28 and negative for 39 samples. Twenty samples were positive and 34 were negative by both methods. The overall sensitivity of LRP assay was 80% (20/25) and specificity 81% (34/42) (Table 5.1). Time to detection ranged between one to four

weeks with a median at 2.3 weeks by LJ and that of LRP ranged between 1 to 12 days with the median at 5.7 days.

Table 5.1: Comparison of optimized LRP with conventional LJ method

LRP	LJ		
		POS	NEG
POS	20	8	28
NEG	5	34	39
TOTAL	25	42	67

Conclusion:

Optimized LRP assay shows promise as a rapid assay for diagnosis of TB from sputum samples. Further evaluation with more number of samples can be done to include it as a standard diagnostic test.

(PI: Dr.Vanaja Kumar; vanajakumar@trchennai.in)

Application of LRP assay for screening compounds from natural sources for antimycobacterial activity

Background:

The resurgence of TB is one of the most serious public health challenges of the 21st century. The need for discovering novel antimycobacterial compounds has been strongly felt due to the emergence of strains resistant to classic anti-TB drugs. Natural and semisynthetic compounds from actinomycetes and plants have been shown to have high levels of antimycobacterial activity. LRP assay has been employed for rapid screening of such compounds for antimycobacterial activity because of its simplicity and economy.

Aim:

- To study the effectiveness of nine extracts from extreme ecosystem actinomycetes and 25 plant-derived chalcone semisynthetic compounds against strains of *M.tuberculosis*

Methods:

Laboratory and clinical strains of *M. tuberculosis* were grown in compound-containing and compound-free Middlebrook 7H9 complete medium for three days at 37°C. Concentrations of 50 and 100 µg/ml were tested for chalcone derivatives while actinomycetes extracts were tested in 100 and 500 µg/ml concentrations. Rifampicin was included as assay control. LRP phAE129 was added and the samples were incubated for three hours. Equal volume of the cell phage mixture was mixed with 0.3 mM D-Luciferin in 0.05M sodium citrate buffer of pH 4.5. Light output was immediately measured as RLU in the luminometer at 10 seconds integration. Compounds exhibiting a reduction of 50% or more in RLU in the test vials compared to that of the control were considered to have antimycobacterial activity.

Results:

Among nine extracts of actinomycetes, four were found to have antimycobacterial activity at a concentration of 100 µg/ml while six of them inhibited growth at a concentration of 500µg/ml (Table 5.2). Out of the 25 Chalcones derivatives, 10 and 17 compounds showed antimycobacterial activity at a concentration of 100 µg/ml and 500 µg/ml respectively (Table 5.3).

Table 5.2: Antimycobacterial activity of Actinomycetes

Extract	% reduction in RLU	
	100 µg/ml	500 µg/ml
D10- A	84.65	87.65
D10- B	84.17	85.97
D10 –C	79.02	81.77
D5	0	88.13
K29	0	0
KA1	26.98	85.25
ANS2	64.15	78.18
AD10	0	0
K24 A	0	0
R (2 µg/ml)	84.41	

Table 5.3: Antimycobacterial activity of chalcone derivatives

Compounds	% reduction in RLU	
	50 µg/ml	100 µg/ml
C1	70.02	70.18
C2	46.29	66.51
C3	30.95	48.06
C4	41.93	50.64
C5	31.59	98.86
C6	7.36	17.38
C7	68.41	90.52
C8	16.88	51.59
C9	24.06	48.83
C10	0	0
C11	58.75	65.08
C12	0	0
C13	66.07	72.98
C14	0	21.87
C15	43.16	63.9
C16	29.25	55.16
C17	0	12.17
C18	92.19	96.45
C19	64.61	89.27
C20	90.06	96.22
C21	94.3	98.15
C22	31.55	33.8
C23	60.24	98.52
C24	98.9	99.04
C25	84.16	97.94
R (2 µg/ml)	84.41	

Conclusion:

Actinomycetes and chalcone derivatives have moderate to high inhibitory activity against *M. tuberculosis*. They may be ideal candidates for development of anti-TB agents.

(PI: Dr. Vanaja Kumar; vanajakumar@trchennai.in)

Use of phagebiotics for growing tubercle bacilli in liquid medium from sputum processed by Chitin and Petroff's methods**Background:**

Growing *M. tuberculosis* in liquid medium is mandatory for rapid diagnosis of TB. Overgrowth of normal flora escaping the action of sputum processing chemical is the major problem in liquid media used for the primary isolation of tubercle bacilli, which affects the sensitivity of any rapid assay. Phage cocktail (Phagebiotics) substituting the use of antibiotics to control the overgrowth of normal flora in sputum samples has been established and it forms a novel, bio-friendly approach to tackle the problem of non-mycobacterial contaminants. It is essential to know the effect of phagebiotics on the growth and retrieval of *M. tuberculosis* to use it in rapid diagnostic assays.

Aim:

- To evaluate the effect of phagebiotics on sputum samples processed by Chitin and modified Petroff's methods for the early and better recovery of tubercle bacilli on LJ medium

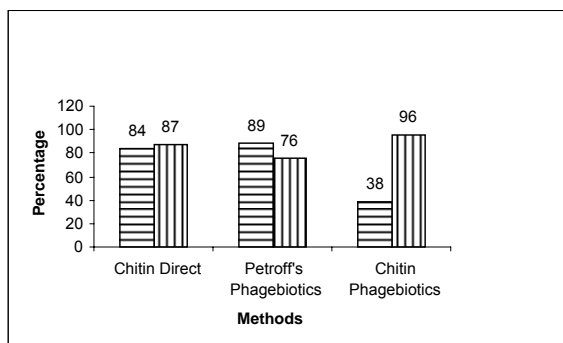
Methods:

A total of 120 sputum samples were collected from Otteri TB hospital, Chennai and divided into two parts and randomized. One part was processed by modified Petroff's method and other by Chitin method. One loopful each of the sputum deposits were inoculated onto two LJ medium slopes, two ml of phagebiotics was added to rest of the sputum deposits and incubated at 37°C for 18-24 hours. Later, one loopful from each of the deposits was inoculated onto two LJ slopes, randomized, incubated at 37°C and read every week up to eight weeks.

Results:

Culturing *M. tuberculosis* after processing by modified Petroff's method is considered as gold standard. Culture results of chitin direct, chitin-phagebiotics and Petroff's-phagebiotics were compared with Petroff's culture. Sensitivity and specificity of Petroff's-phagebiotics was 89% and 76% respectively while that of chitin direct method was 84% and 87% respectively (Fig 5.4). Only 38% of the real positives were picked up by chitin-phagebiotics while the specificity was as high as 96%. Chitin being acidic, sputum deposits processed with chitin had a pH of ~ 5.5 after the addition of phagebiotics while those processed with 4% sodium hydroxide were at ~ 8.5. Overnight incubation of sputum deposits in acidic pH probably lead to the killing of tubercle bacilli.

Fig. 5.4: Sensitivity and specificity of culture methods in comparison with Petroff's method



Conclusion:

Sputum processing with chitin resulted in moderately better retrieval of tubercle bacilli, while overnight incubation with phagebiotics reduced the sensitivity much further. Reduction in pH and dilution of reaction mixture by the addition of phagebiotics lead to the reduction in number of viable bacilli. Further studies are planned with neutralized deposits.

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Evaluation of nitrate reductase assay as rapid inexpensive method for identification of multi drug resistant tuberculosis

Background:

Multi drug resistant TB is an increasing public health concern globally, especially in low-income countries. Current DST methods for *M. tuberculosis* are either expensive and automated or time consuming and conventional. There is a great need for a rapid, reliable and inexpensive method for DST.

Aim:

- To compare the nitrate reductase assay (NRA), a colorimetric method based on the ability of *M. tuberculosis* to reduce nitrate to nitrite

Methods:

A panel of 100 *M. tuberculosis* strains with various resistance patterns was tested using the NRA. Minimal inhibitory concentration (MIC), proportion sensitivity test (PST) and BACTEC 460 methods were used for comparison. Results of NRA were read after incubation for 7 to 14 days.

Results:

The susceptibility results of 100 *M. tuberculosis* strains against Sm, H, R, E and ofloxacin (O) gave an overall agreement of 91% between the NRA and BACTEC - 460, 95% for PST and 92% for MIC. The sensitivity of NRA compared with BACTEC - 460, PST and MIC was 98%, 98%, 100% for H, 97%, 98%, 98% for R. The specificity was 98%, 96% and 96% for H, 95%, 98% and 98% for R. For majority of cases, the results were available in 10 days.

Conclusion:

This study showed that the NRA method is rapid and inexpensive and could correctly identify most resistant and sensitive *M. tuberculosis* strains. The agreement between NRA and other methods suggests that this test has the potential to become an alternative for rapid and accurate detection of MDR-TB, particularly in resource poor settings.

(PI: Dr. Ranjani Ramachandran; ranjanir@trchennai.in)

Characterization of HIV-1 isolates from antiretroviral naïve children in south India

Background:

Although HIV/AIDS can affect people of all ages, this epidemic has shown a rapid shift towards women and young people, with about half of the new infections occurring in ages below 25. Knowledge about the prevalence and pattern of HIV drug resistance in the community is important for formulating ART strategies for countries/populations. Most studies of antiretroviral drug resistance have been in adults; however, these results may not be directly applicable to pediatric cohorts, since factors that influence selection of drug resistant variants may be different in children. The factors responsible for such variations include the heterogeneity of HIV-1, the pharmacokinetics of antiretroviral drugs across different age groups, and unique barriers to adherence to treatment regimens.

Aims:

- To characterize the distribution of HIV-1 subtypes among treatment-naïve children in Chennai, south India
- To describe polymorphisms in reverse transcriptase (RT) and protease genes
- To detect the presence of drug resistant mutations if any

Methods:

Pattern of polymorphism and potential drug resistance mutations were evaluated in HIV-1 isolates from 48 children naïve to ART, attending the outpatient clinics of the TRC. The samples were subjected to genotyping of RT and protease genes.

Results and conclusion:

All the isolates were identified to be HIV-1 subtype C. All samples showed significant polymorphisms in both RT and protease genes, but none had major drug resistance mutation. Hence, the currently recommended generic first line antiretroviral drug combination is an appropriate treatment strategy for ART naïve HIV-1 infected children in India.

(PI: Dr.Soumya Swaminathan; soumyas@trcchennai.in.)

Study of *CYP2B6* polymorphism (G516T) and *ABCB1* polymorphism (C3435T) in HIV-1-infected individuals from south India

Background:

The NNRTIs, EFV and NVP are used as a first-line treatment of HIV-infected patients. Plasma concentrations of EFV and NVP are known for a high degree of inter-patient variability. These variations could be due to single nucleotide polymorphisms in genes coding for certain drug-metabolising enzymes or transporter proteins. A G to T change at position 516 (that is, 516 G>T) of *CYP2B6* gene and a C to T change at position 3435 of *ABCB1* gene could influence plasma concentrations of EFV and NVP. We studied the influence of *CYP2B6* G516T and *ABCB1* C3435T gene polymorphisms on EFV and NVP plasma concentrations in HIV-infected patients in south India, and also the prevalence of this polymorphism in the same population.

Methods:

The study participants comprised of HIV-infected individuals and apparently healthy subjects. The HIV-infected persons were receiving ART with EFV or NVP-based HAART regimens from the Government Hospital of Thoracic Medicine, Tambaram, Chennai. The patients were receiving EFV (600 mg once daily) or NVP (200 mg bi-daily) along with 3TC (150 mg bi-daily) and stavudine (d4T) (30/40 mg bi-daily)/zidovudine (AZT) (300 mg bi-daily). None of the patients was receiving any co-medications that would alter the blood levels of EFV or NVP. The healthy subjects consisted of staff working at the centre.

Venous blood (2 ml each) was collected in both heparin - and ethylene diamine tetra acetic acid (EDTA) - containing vacutainers from all the patients, while in the case of healthy subjects, blood was collected only in EDTA vacutainers. The time of blood collection was chosen in such a way that it represented 12-hour concentration for EFV and 2-hour concentration for NVP. The EDTA sample was used for DNA extraction and genotyping of *CYP2B6* and *ABCB1* polymorphism by PCR-RFLP analysis. The different genotypes of *CYP2B6* (GG, GT & TT) and *ABCB1* (CC, CT & TT) were identified based on the number and size of the fragments. Estimation of EFV and NVP in heparinised plasma was undertaken by high performance liquid chromatography (HPLC).

Results:

CYP2B6 G516T polymorphism: A total of 184 subjects took part in the study (130 HIV-1-infected persons and 54 healthy subjects). Of them, 50 were of the GG genotype (27%), 63 belonged to the GT genotype (34%) and 71 belonged to the TT genotype (39%). The G and T allele frequencies were 0.44 and 0.56 respectively.

Among the 130 HIV-infected persons, 64 were receiving treatment with EFV and 66 with NVP-containing regimens. The mean 12-hour plasma EFV concentration in the GG, GT and TT genotypes were 1.85, 2.0 and 6.44 µg/ml respectively (Table 5.4). The TT genotypes had a significantly higher plasma EFV concentration than the GG and GT genotypes ($p < 0.01$). The mean 2-hour NVP blood levels for GG, GT and TT genotypes were 7.6, 7.95 and 10.53 µg/ml respectively (Table 5.4). The plasma NVP concentrations were significantly higher in the TT genotype compared to GG and GT genotypes ($p < 0.05$).

ABCB1 C3435T polymorphism: This polymorphism was studied in a total of 179 subjects (126 HIV-1-infected persons and 53 healthy subjects). Of the 179 subjects, 78 were of the TT genotype (44%), 74 belonged to the CT genotype (41%) and 27 belonged to the CC genotype (15%). The C and T allele frequencies were 0.36 and 0.64 respectively.

Among the 126 HIV-infected persons, 55 were receiving treatment with EFV and 71 with NVP-containing regimens. The mean 12-hour plasma EFV concentration in the CC, CT and TT genotypes were 5.22, 3.5 and 2.48 µg/ml respectively (Fig. 5.5a). Although plasma EFV concentrations were highest in the CC genotype, followed by CT and TT genotypes, the differences failed to attain statistical significance. The mean 2-hour NVP blood levels for CC, CT and TT genotypes were 8.33, 8.99 and 7.5 µg/ml respectively (Fig. 5.5b). The plasma NVP concentrations were lower in the TT genotype compared to CC and CT genotypes. However, none of the differences was statistically significant.

Conclusions:

The study data show that frequency of *CYP2B6* G516T and *ABCB1* C3435T polymorphisms are high in the ethnic south Indian population. TT genotypes of the *CYP2B6* polymorphism have elevated plasma concentrations of EFV and NVP. With respect to *ABCB1* C3435T polymorphism, a trend in plasma EFV

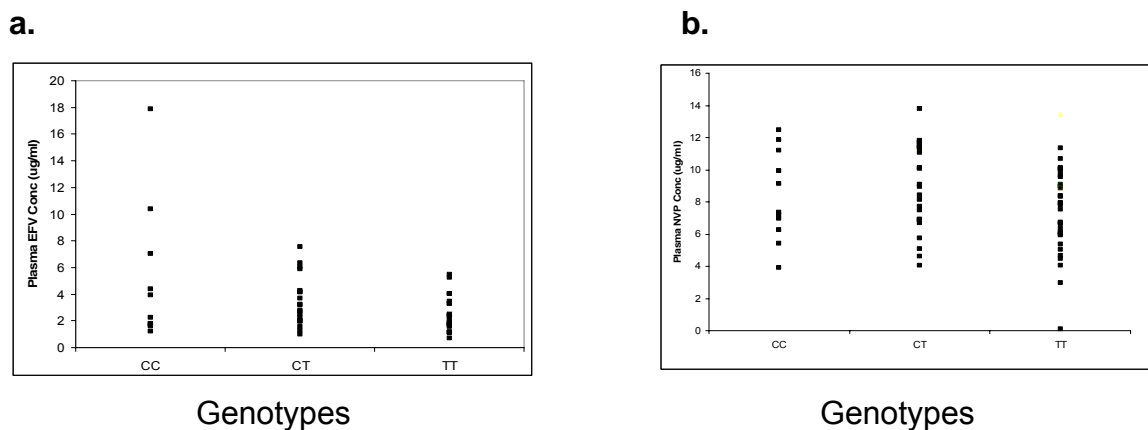
concentrations was observed; patients with the CC genotype having the highest values followed by CT and TT genotypes. These findings suggest that inter-individual variations in plasma concentrations of EFV and NVP could be due to genetic variations in the *CYP2B6* gene and to a lesser extent *ABCB1* gene.

Table 5.4: Influence of *CYP2B6* polymorphism on plasma EFV & NVP

Mean \pm SD ($\mu\text{g/ml}$)		
Genotype	12-hr EFV (n=64)	2 hr-NVP (n=66)
GG	1.85 \pm 1.15 (18)	7.60 \pm 1.59 (19)
GT	2.00 \pm 0.72 (19)	7.95 \pm 2.82 (22)
TT	6.44 \pm 2.63* (27)	10.53 \pm 2.64* (25)

p < 0.05 vs GG & GT
n given in parantheses

Fig. 5.5: Plasma concentrations of EFV (a) and NVP (b) in the different genotypes of *ABCB1* C3435T



(PI: Dr. Geetha Ramachandran; geethar@trcchennai.in)

Effect of anti-coagulants on plasma nevirapine and efavirenz concentrations

Background:

Plasma concentrations of the NNRTIs, NVP and EFV are reported to correlate with antiviral effect and possibly toxicity. Heparin is routinely used as an anticoagulant for plasma drug estimations, while EDTA is used for viral load and CD4 cell count measurements. Use of EDTA plasma for drug estimations, if found suitable, would have several practical advantages. Most of the developing countries do not have state-of-the-art sample collection and/or storage capabilities. Hence more data are needed on how different anticoagulants influence NVP and EFV concentrations.

Aim:

- To compare NVP and EFV concentrations in paired heparin and EDTA plasma in HIV-infected individuals undergoing ART

Methods:

The study was conducted in HIV-infected individuals who were receiving ART at the Government Hospital of Thoracic Medicine, Tambaram, Chennai. Their treatment consisted of NVP or EFV along with 3TC and d4T/AZT. Blood draws were made at the same time in heparin and EDTA vacutainers. Plasma concentrations of NVP and EFV were estimated according to validated methods by HPLC.

Results:

One hundred HIV-infected patients took part in the study. Of them, equal numbers were receiving NVP and EFV. The time of blood collection was at 2 and 12 hours respectively after NVP and EFV drug intake. The mean NVP concentrations in heparinised and EDTA plasma were 8.79 and 8.59 µg/ml respectively (Table 5.5). This difference was not statistically significant ($p=0.203$). In the case of EFV, the corresponding values were 3.03 and 2.78 µg/ml respectively (Table 5.5); the difference was statistically significant ($p<0.05$); However, the percent variation was less than 10%.

Table 5.5: Efavirenz and Nevirapine concentrations in Heparin and EDTA plasma

Variables	Nevirapine (n = 50)		Efavirenz (n = 50)	
	Heparin	EDTA	Heparin	EDTA
Mean	8.79	8.59	3.03	2.78
SD	3.16	3.02	3.08	2.79
Median	9.02	8.88	2.03	1.88
Range	0.10 – 14.82	0.12 – 15.36	0.11 – 16.22	0.11 – 12.65
P	0.203 (NS)		< 0.05 (S)	

Conclusion:

Plasma NVP levels did not differ when heparin or EDTA was used as anticoagulant. However, in the case of EFV, the difference between heparin and EDTA plasma was significant. Hence for plasma NVP determination, blood collected in heparin or EDTA vacutainers could be used.

(PI: Dr. A.K. Hemanth Kumar; hemanthkumarak@trcchennai.in)

Steady state pharmacokinetics of efavirenz in HIV-infected patients in south India**Background:**

Concomitant administration of HAART and anti-TB medications is often difficult because of drug-drug interactions and the adverse effects profile. Efavirenz, a NNRTI has been recommended as a first line option in ART and the preferential choice in TB and HIV co-infected patients. The dosage of EFV when co-administered with R is a matter of debate.

Aim:

- To study the influence of sex, body weight, CYP2B6 G516T polymorphism and R co-administration on the steady state pharmacokinetics of EFV in HIV-1-infected patients in south India

Methods:

Thirty four HIV-1-infected patients (25 males; 9 females) receiving ART with EFV (600mg once daily)-containing regimen at the Government Hospital of Thoracic Medicine, Tambaram, Chennai were recruited into the study. Of them, 15 had active TB and were receiving treatment with R-containing regimens (450/600 mg thrice weekly). The HIV-TB co-infected patients were investigated on two occasions, while receiving ART and ATT, and at least one month after stopping ATT. Serial blood collections predosing and at 1, 2, 4, 6, 8, 12 and 24 hours post dosing were collected in heparinised vacutainer tubes. An additional sample was collected in an EDTA vacutainer tube at any time point. Plasma EFV was estimated in the heparin samples by HPLC. The EDTA blood sample was used for DNA extraction and genotyping of *CYP2B6* G516T polymorphism by PCR-RFLP analysis.

Results:

Males and females did not significantly differ in peak concentration (C_{max}), trough concentration (C_{min}) and exposure (AUC_{0-24}) of EFV. Patients with body weight > 50 kg (n=14) had lower C_{max} (5.5 vs.10.5 $\mu\text{g/ml}$), C_{min} (2.4 vs.6.2 $\mu\text{g/ml}$) and AUC_{0-24} (78.4 vs.176.4 $\mu\text{g/ml.h}$) than those with body weight \leq 50 kg (n=20); these differences were not statistically significant. C_{max} , C_{min} and AUC were significantly higher in TT genotypes than GT and GG genotypes ($p < 0.05$). Rifampicin co-administration caused significant reduction in C_{max} , C_{min} and AUC_{0-24} ($p < 0.05$), the mean percent decreases being 26%, 29% and 25% respectively (Table 5.6). Three out of 15 patients had C_{min} below sub-therapeutic level (<1 $\mu\text{g/ml}$) in the presence of R.

Conclusions:

CYP2B6 G516T polymorphism and R co-administration significantly influence the pharmacokinetics of EFV. Although R co-administration and higher body weight caused reduction in plasma EFV concentrations, dose enhancement of EFV may not be necessary since the C_{min} of EFV was above 1 $\mu\text{g/ml}$, that is, the drug concentrations did not drop to sub-therapeutic levels in majority of the patients studied.

Table 5.6: Steady state pharmacokinetics of EFV with and without R

Patients n=15	C _{max}	C _{min}	T _{max}	AUC ₍₀₋₂₄₎	Cl	T _{1/2}
EFV	9.33 ± 8.04	5.28 ± 6.33	2.60 ± 1.06	156.04 ± 174.72	6.62 ± 4.12	24.98 ± 13.19
EFV + R	6.86 ± 6.52	3.76 ± 5.21	2.47 ± 0.99	116.52 ± 135.69	10.26 ± 6.08	17.57 ± 9.42
% decrease	26.5%	28.8%	--	25.3%	55.0%*	--
p value	<0.05	<0.05		<0.05	<0.05	<0.05

*percent increase;

C_{max} – Peak concentration; C_{min} – Trough concentration; T_{max} – Time to attain C_{max}; AUC₍₀₋₂₄₎ – Exposure; Cl – Clearance; t_{1/2} – Half-life

(PI: Dr. Geetha Ramachandran; geethar@trchennai.in)

Ongoing Studies:

Detection of mycobacterial species by high performance liquid chromatography

Background:

Identification of mycobacteria has traditionally been based on growth characteristics and a battery of time-consuming biochemical tests. Unfortunately, the specificity and sensitivity of these tests in recognizing new species are low. Only genetic and chromatographic analyses seem to have the potential to recognize new species of mycobacteria. Reverse phase-HPLC of high molecular weight mycolic acids are rapid and unlike genetic probes are not limited to the identification of only a few species. Non-tuberculous mycobacteria (NTM) have been reported to cause localized or disseminated disease especially in HIV patients. This was particularly true in case of *M. avium* complex (MAC), because these organisms are the single most important cause for disseminated bacterial infection in AIDS patients.

Aims:

- To identify mycobacterial species from primary culture isolates by HPLC
- To assess the prevalence of NTM species among these isolates

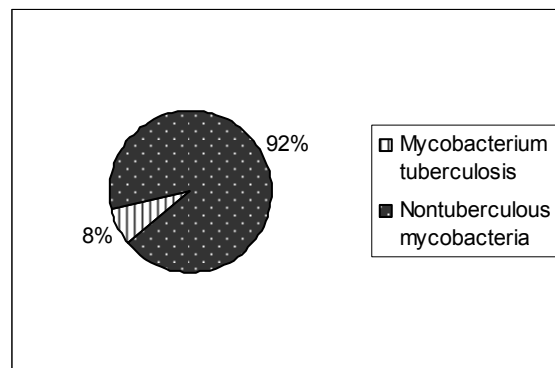
Methods:

Sputum samples referred to TRC during the year 2006 were included in the study. The cultures with atypical morphology or cultures with discordant results by biochemical tests were subjected to HPLC for species level identification. The parameters set for the HPLC system, chemicals and solvents, testing procedures were followed as per Centers for Disease Control (CDC), USA protocol. A set of standard strains were processed along with the specimens and used as controls for chromatographic pattern comparison.

Results:

Out of 212 isolates, 179 were subjected for species level identification by HPLC among which 165 (92%) were identified as NTM and 14 (8%) as *M. tuberculosis* (Fig. 5.6). The predominant NTM species were found to be *M.kansasii* (28%), followed by *M.avium* (15%), *M.fortuitum* complex (12%), *M.intracellulare* (9%) and *M.parafortuitum* (8%).

Fig.5.6: Results of HPLC analysis



(PI: Dr.Ranjani Ramachandran; ranjanir@trcchennai.in)

Innate and adaptive immunity in children starting antiretroviral drugs in India

Background:

Globally, more than 2000 children acquire HIV infection daily from their mothers, 90% of whom reside in developing countries. Despite the well-known benefits of ART for the treatment of AIDS, access to therapy has been a problem for the majority of HIV-infected children in the world. In India, the government has recently initiated a program to provide free ART to children below 15 years of age. There is a need to gather pediatric specific clinical data and laboratory indicators of the risk of progression during the first few years of life.

Aim:

- To identify novel laboratory markers that provide insights into functional immune status of HIV-infected children that are better than assays of CD4 positive T cells and plasma viral load.

Methods:

This project will enroll HIV infected children (birth -13 years) who will be given ART as per standard of care based on national guidelines of India and who will be monitored for immunologic and virologic status by routine and proposed novel markers of innate and adaptive immunity. These assays include studies of dendritic cell number and function and surface markers on CD4 and CD8 positive T cells. Patient enrollment to this study started in February 2007. So far, 23 children have been enrolled into the study.

(PI: Dr. Soumya Swaminathan, : soumyas@tgrcchennai.in; Funding: Indo-US JWG Maternal and Child Health (NIH and ICMR)

Therapeutic drug monitoring of nevirapine and efavirenz in HIV-infected children in India

Background:

Highly active antiretroviral therapy has resulted in an impressive reduction in the rate of disease progression and death in HIV-1 infected children. Antiretroviral drugs for pediatric use have been very recently introduced by the Government of India and are made available at the Government ART centres. Limited information is available on the blood levels of NVP and EFV in children receiving the pediatric formulations in India.

Aim:

- To estimate the trough and peak levels of NVP in HIV-infected children receiving treatment with generic pediatric formulations and examine if drug levels are within the therapeutic range of the drugs (In the case of children receiving EFV-based regimens, the 12-hour drug level will be studied)

Methods:

HIV-infected children receiving treatment at the Government ART centres at the Government Rajaji Hospital, Madurai, B.J.Wadia Hospital, Mumbai, Government Hospital of Thoracic Medicine, Tambaram and Kilpauk Medical College and Hospital, Chennai will form the study population. Children aged 6 months to 14 years, receiving treatment regularly with generic antiretroviral drugs as fixed dose combinations (FDCs) based on body weight/body surface area, for at least two weeks will be recruited. It has been proposed to include about 100 children in the study. Blood samples before and at two hours after drug administration will be collected. In the case of EFV, a single blood sample will be collected. Plasma NVP and EFV estimations will be carried out by HPLC. The blood levels of NVP and EFV obtained in children will be examined to see if they fall within the therapeutic range of the drugs (3 – 12 µg/ml for NVP and 1 – 4 µg/ml for EFV). So far, 58 and 11 children receiving NVP and EFV respectively have been included into the study. The study is in progress.

(PI: Dr. Soumya Swaminathan; : soumyas@trcchennai.in)

Feasibility of monitoring antiretroviral treatment adherence using urine and salivary lamivudine: a study in healthy volunteers

Background:

Adherence to ART is a strong predictor of virological suppression, disease progression and death. Although currently available approaches to measure

adherence have proved to be predictive of outcomes, the results are variable. Some investigators have assessed the antiretroviral drug levels in blood as a measure of adherence. Alternatively, urine or saliva could serve as useful biological fluids for detecting antiretroviral drug levels particularly to monitor patient adherence to treatment if found feasible. Lamivudine, a cytosine nucleoside analogue has been effectively used in combination with other antiretroviral drugs to treat HIV-1 infection. Its levels in urine or saliva could be used to study patient adherence to treatment.

Aims:

- To estimate the concentration of 3TC excreted in urine collected at different time points between 0 – 24 hours of drug administration
- To estimate the concentration of 3TC in paired plasma and saliva collected at different time points after drug administration

Methods:

The study is being carried out in collaboration with Department of Pharmacology, Madras Medical College, Chennai. Twelve healthy male volunteers will take part in the study. On the day of the study, a sample of blood (three ml) will be collected in a heparinised vacutainer. One tablet of 3TC (150 mg) will be administered with 200 ml water. Blood samples will be collected at 1, 2, 4, 6, 8, 12 and 24 hours of drug administration. They will be instructed to collect about 3 ml of saliva at the same time points. Complete urine collections made between 0-8, 8-16 and 16-24 hours will be collected in separate containers. Lamivudine concentrations in plasma, saliva and urine will be estimated by HPLC. Based on plasma and salivary concentrations of 3TC at different time points, certain pharmacokinetic variables will be calculated. The percent dose of 3TC excreted in urine collected at different time periods will be calculated.

So far, eight subjects have been recruited into the study. The study is in progress.

(PI: Dr. Geetha Ramachandran; geethar@trcchennai.in)

Multicentric evaluation of a sensitive smear microscopy technique for detection of AFB in sputum

Background:

The smear method is essentially a component of a multipurpose method that is applicable on both pulmonary and extra-pulmonary specimens and also compatible with culture and PCR techniques. This method is likely to identify a significant number of patients who go undetected by the direct method of smear microscopy thereby contributing to improved disease control.

Aim:

- To evaluate a highly sensitive method of smear microscopy developed in Dr. Jaya S. Tyagi's laboratory at All India Institute of Medical Sciences, New Delhi in a multicentric laboratory setting

Methods:

Sputum samples collected from Otteri TB Hospital are transported to the TRC Bacteriology laboratory and processed within 4 to 6 hours. A maximum of 8 – 10 samples are processed per day. After receiving the sputum sample direct smear is made. Later each sample is divided into two portions of approximately equal volume. One portion is processed by modified Petroff's method and the other portion by the universal sample processing (USP) method for culture. The intake is continuing and about 800 samples are processed.

(PI: Dr.N.Selvakumar; : selvakumarn@trcchennai.org. Funding: WHO/ICMR)

Standardization of second line anti-TB drug susceptibility testing

Background:

The global situation with respect to TB has worsened with the emergence of MDR-TB. Various studies have shown that MDR-TB can be cured by a combination of second-line drugs under DOTS-Plus. With the spread of MDR-TB, there is an increasing demand for DST for second line drugs. Since the critical concentration for second line drugs have not been completely established, the present study is focusing on evaluating the critical concentration of some of the second line drugs namely kanamycin (K), ethionamide (Eth), capreomycin (C), amikacin (A), PAS and Z. Previously established critical concentrations for

second line drugs using conventional methods will be performed and compared with automated systems and other rapid phenotypic methods. After establishing the critical concentrations, the results will be analyzed for overall correlation between the various methods used.

Aims:

- To standardize DST of second line drugs using standard conventional methods and automated systems and other rapid phenotypic methods
- To compare the results obtained by automated and phenotypic rapid methods with that of standard conventional methods

Methods:

Clinical isolates from the patients attending the TRC clinic will be selected for this experiment. Selection of isolates are based on their first line drug susceptibility pattern, where 50% of them are MDR and 25% were polyresistant to first line drugs and 25% fully susceptible to all drugs. Isolates freshly subcultured onto LJ medium will be used for setting up DST after randomization. The following methods for DST will be used viz: LJ, agar, liquid medium, automated systems and colorimetric methods.

(PI: Dr.Ranjani Ramachandran : ranjanir@trcchennai.in Funding: WHO-USAID)

Microscopic observation drug susceptibility assay as a rapid low cost test for detection and drug susceptibility of *Mycobacterium tuberculosis* in HIV-TB and non HIV-TB Individuals

Background:

In the last decade, there has been dramatic resurgence in the incidence of TB throughout the world. The situation is compounded with increasing HIV infections. India is estimated to have 3.5 million HIV patients and about 1.8 million of them are co-infected with TB. Most cases occur in developing, resource poor countries, where the expensive automated systems (MGIT 960 and BACTEC 460) are not feasible. To curb the transmission of infection early detection of MDR-TB using a low cost rapid test is the need of the hour. The principle of the microscopic observation broth–drug susceptibility (MODS) test is

based on the microscopic detection of *M. tuberculosis* as strings or cords in Middlebrook 7H9 broth medium with or without antimicrobial agents.

Aims:

- To evaluate MODS for the detection and drug susceptibility of *M. tuberculosis* in sputum samples from patients with suspected pulmonary TB in HIV positive and non-HIV individuals
- To compare the results of MODS with conventional methods and automated systems to detect cross contamination and relative cost

Methods:

The sputum samples referred to TRC will be processed for AFB culture as per standard protocol. The remaining sputum sediment will be inoculated into 7H9 broth with and without drug and periodically examined for cord formation under inverted microscope. DST is performed for the following drugs: Sm, H, R, E, K, Eth and ofloxacin (O). The results of MODS after decoding will be subjected to statistical analysis.

(PI: Dr.Ranjani Ramachandran : ranjanir@trchennai.in Funding: WHO-USAID)

Tetrazolium micro plate assay - Rapid colorimetric method and thin layer agar for determination of DST of *M. tuberculosis*

Background:

Tuberculosis has acquired a growing importance in developed and developing countries with the emergence of MDR-TB. To control the spread of MDR-TB rapid and reliable method for early diagnosis and rapid DST is the need of the hour. There are various rapid methods available for DST, which includes, automated system using BACTEC 460 and MGIT 960 (Becton and Dickenson) and molecular methods such as INNO-LiPa (Line probe assay) that are expensive and are impractical for routine use. In the last few years much attention has been posed towards colorimetric assays such as NRA using solid media, micro alamar blue assay (MABA) and tetrazolium microplate assay (TEMA), which are quite rapid and cost effective and easy to perform.

Aims:

- To standardize and evaluate TEMA and thin layer agar (TLA)
- To determine susceptibility of *M.tuberculosis* for first and second line drugs
- To compare the TEMA and TLA results with that of conventional methods

Methods:**Samples size:**

One hundred and thirty cultures from patients referred to TRC were selected for the study and randomized by statistician.

TEMA: A rapid colorimetric method based on the principle of reduction of MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5 diphenyl tetrazolium bromide] for determination of susceptibility to the anti-TB drugs (Sm, H, R, E, K, Eth and O).

Culture suspension was prepared in 7H9 GC broth and tested for drug susceptibility using 96 well microtitre plate for the above mentioned anti-TB drugs. After incubation readings will be taken on days 5, 7, 9, 11, 13 and 15 respectively. Resistance is determined by the development of purple colour and susceptibility by no colour change in the appropriate drug concentrations.

TLA: This method is capable of detecting growth within 9-11 days. Initial identification of *M. tuberculosis* based on colony morphology in solid medium when viewed microscopically can be assessed.

Culture suspension prepared in 7H9 broth and 10 fold dilutions will be prepared (S1, S2, S3 & S4). Drug susceptibility testing is performed for the following drugs (Sm, H, R, E, K, Eth and O).

After incubation readings will be taken on days 3, 5, 7 and 10 respectively under an inverted light microscope. Susceptibility pattern can be determined by detecting microcolonies in drug free and drug containing plates. Percentage of resistance is calculated. After completion of the experiments, the strains will be decoded and results will be analyzed using appropriate statistical method.

(PI: Dr.Ranjani Ramachandran : ranjanir@trcchennai.in Funding: WHO-USAID)