

Applied Research

Studies completed:

Phagebiotics to process sputum specimens for Luciferase Reporter Phage assay

Background:

The mechanical pressure during centrifugation and the chemical pressure due to the alkali leave the tubercle bacilli in the sputum unsuitable for the phage to infect soon after processing sputum specimens by Petroff's procedure. It is mandatory that sputum specimens processed by 4% NaOH in Petroff's method have to be incubated overnight before subjecting it to any phage infection. Incubation overnight even with antibiotics yields heavy growth of normal flora that survives the action of 4% NaOH making it unsuitable for phage assay.

Aim:

To isolate normal flora that survives the action of 4% NaOH, isolate phages which infect all of them, add them to sputum deposits and assess their capacity to control the overgrowth of normal flora.

Methods:

The flora that survived the action of 4% NaOH was identified and 14 representative isolates were selected. Using them as bait, a total of 8 phages were isolated from soil and sewage

samples. The host range of all these phages were assessed after preparing high titre phage lysates. Three of them inhibited 13/14 clinical isolates belonging to different genera including aerobic spore bearers excepting *Staphylococcus albus*. None of the other phages could control *Staphylococcus albus*. Screening more sewage samples is being attempted to get a suitable lytic phage infecting *Staphylococcus albus*.

Results:

Using the three phages with the wide host range as a cocktail, 100 sputum samples were analysed. Processed sputum samples when grown in liquid medium to facilitate rapid diagnosis of TB, yielded heavy growth of normal flora when subcultured onto blood agar plates. Addition of phage cocktail resulted in qualitative and quantitative reduction in commensal flora.

Conclusion:

The use of phagebiotics to control the overgrowth of normal flora when sputum specimens are incubated overnight is a biofriendly approach most suited for Luciferase Reporter Phage (LRP) diagnostic assay. However the present set of phagebiotics should be strengthened further to control *Staphylococcus albus* and *Bacillus sp.* (Table V).

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Table V: Growth of normal flora on blood agar from 100 sputum samples processed by Petroff's method as such (stage I) and after growing in liquid medium with (stage III) and without phagebiotics (stage II)

	Stage I	Stage II	Stage III
Hemolytic, Nonhemolytic Confluent	2	100	4
Nonhemolytic Confluent	11	0	0
Hemolytic, Single	4	0	17
Non hemolytic, Single	9	0	33
No Growth	74	0	46

Increasing nevirapine dose can overcome reduced bioavailability due to rifampicin co-administration

Background:

Majority of patients in the developing world who are receiving antiretroviral therapy are on 3-drug fixed dose combination pills that include nevirapine. Rifampicin, an integral part of anti-TB therapy, induces the cytochrome P-450 system, which is involved in the metabolism of nevirapine. This could lower the bioavailability of nevirapine when it is given along with rifampicin. Although it has been suggested that rifampicin could be administered along with nevirapine, supportive pharmacokinetic data of nevirapine in the presence of rifampicin are scarce.

Aim:

To study the effect of rifampicin on steady state pharmacokinetics of nevirapine and the impact of increasing the dose of nevirapine on its peak and trough levels.

Methods:

Thirteen HIV-infected patients (9 males and 4 females) with mean age of 34 yrs and body weight 58 kg and on regular antiretroviral therapy for a

period of 1–8 months (stavudine 30/40 mg + lamivudine 150 mg + nevirapine 200 mg twice daily) participated in the study. A baseline pharmacokinetic study of nevirapine was conducted and repeated after one week of daily rifampicin 450/600 mg. The study was repeated in 7 out of 8 patients who had sub-therapeutic trough nevirapine levels, after increasing their nevirapine dose to 300 mg twice daily. Liver function was monitored.

Results:

The steady state pharmacokinetic parameters calculated based on plasma nevirapine concentrations alone and in combination with rifampicin are given in Table VI. Significant reductions in peak concentration (42%), trough concentration (53%) and exposure (46%) of nevirapine were observed when rifampicin was also administered ($p < 0.01$). The trough concentration of nevirapine fell below the therapeutic range of 3.4 $\mu\text{g/ml}$ in 8 out of 13 patients. An increase of nevirapine dose to 300 mg twice daily raised the trough concentration in all the 7 patients tested to above therapeutic levels and did not cross the toxic level of 12 $\mu\text{g/ml}$. There were no clinical or laboratory adverse events.

Table VI : Steady state pharmacokinetics of nevirapine (n=13)

	Cmax $\mu\text{g/ml}$	Cmin $\mu\text{g/ml}$	AUC(0-12) $\mu\text{g/ml.hrs}$
Nevirapine	8.5 \pm 2.7	5.5 \pm 2.4	80.1 \pm 30.3
Nevirapine + Rifampicin	4.9 \pm 1.7	2.6 \pm 1.4	43.2 \pm 17.3
% Decrease	42	53	46

Cmax - peak concentration
Cmin - trough concentration
AUC - Area under time concentration curve
The above values are Mean \pm SD

Conclusion:

Rifampicin significantly reduced the bioavailability of nevirapine, and the trough concentration to sub-therapeutic levels in a high proportion (62%) of patients. Prospective clinical trials are also required to determine the clinical implications of reduction in nevirapine blood concentrations and the optimal dose of nevirapine to be used in combination with rifampicin.

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Studies in progress

Absorption of nevirapine, lamivudine and stavudine (fixed dose combination) in patients with advanced HIV infection

Background:

The primary aim of antiretroviral treatment is to suppress viral replication. Despite treatment with potent antiretroviral drugs, therapeutic failure during HIV infection could occur due to several reasons. Adequate drug concentrations may not be reaching the virus due to reasons such as poor compliance to treatment, insufficient dosing, poor absorption or drug interactions. This could reduce efficacy and cause HIV resistance to antiretroviral agents. We have demonstrated a significant degree of malabsorption of anti-TB drugs in patients with advanced HIV infection. Information on absorption of generic antiretroviral drugs in HIV-infected patients in India and its relation to the degree of immunosuppression is lacking.

Aim:

To study the absorption of nevirapine, lamivudine and stavudine as a fixed dose combination in patients with varying degrees of

immunosuppression, based on the blood concentrations of the drugs.

Methods:

The study participants comprise of HIV-infected patients undergoing regular antiretroviral treatment with nevirapine 200mg, lamivudine 150mg and stavudine 30/40mg twice daily for a minimum period of two weeks. They will be divided into three groups based on their CD4 lymphocyte counts, i.e. <100 cells, 100 to 200 cells and > 200 cells/mm³. It has been planned to include 12 patients in each group. Steady state pharmacokinetics of nevirapine, lamivudine and stavudine will be determined in all the patients by estimating the drug concentrations in blood collected at different time points following drug administration.

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Nutritional assessment and supplementation in HIV-infected patients with and without tuberculosis

Background:

TB and HIV infection are known to be separately associated with malnutrition and TB might worsen the course of HIV associated immunosuppression and reduce survival among HIV-infected subjects. Despite the high prevalence of TB and malnutrition among HIV seropositive patients, data concerning the nutritional status of TB/HIV co-infected patients in developing countries like India are scarce.

Aims:

1. To document the occurrence of baseline macro and micronutrient deficiencies in HIV-

infected individuals in south India and correlate it with their immune status.

- To test the efficacy of an intervention in the form of a nutritional supplement and to quantitate changes in nutritional, biochemical and immunological parameters over a period of one year.

Methods:

The study commenced in July 2003. The study population included (1) HIV-infected persons without TB and (2) HIV/TB patients who have completed anti-tuberculosis treatment. A baseline clinical, anthropometric and dietary assessment along with laboratory investigations (hematology, biochemistry and immunology) is done for all patients at the time of enrollment to the study. A high calorie, high protein supplement “Indiamix” supplied by the World Food Programme, New Delhi, is given to patients with the advice to consume 100gms per day which supplies an additional 400 Calories and 15 gms of protein. Patients are followed up clinically (including dietary assessment and anthropometric measurement) every 3 months and hematological, biochemical and immunological investigations are repeated every 6 months.

Results:

Five hundred and sixty six patients have been enrolled upto March 2005. This includes 422 asymptomatic HIV-infected individuals and 144 HIV+ve patients (42 females and 102 males) who have completed TB treatment. Two hundred and sixteen patients have completed 12 months of follow up. Ninety percent of our patients are from lower socio-economic strata. The mean age of patients in HIV-TB group was 31.5 ± 6 yrs and in HIV without TB group was 30 ± 7 yrs. Table VII shows the baseline anthropometry and laboratory parameters of the study population. Eighty seven HIV+ve patients have been enrolled as age, sex and socioeconomically matched controls. This group consisted of 48 females and 39 males with their mean age being 31.5 ± 7 yrs. These individuals were not given supplement for first 6 months. Ninety eight age, sex and socioeconomically matched individuals (53 females and 45 males) with mean age of 31 ± 8 yrs, from the community have also been enrolled as non-HIV controls. Table VIII shows the mean nutrient intake for patients in the different study groups.

Patients with HIV infection have lower body weight and Body Mass Index (BMI) than age, sex

Table VII : Baseline Anthropometric and Laboratory Parameters

S.No.	Variables	HIV+ves supplemented with ‘Indiamix’ (Mean \pm S.D.)	
		Treated TB (n=144)	Without TB (n=422)
1)	Weight (kg)	48.0 \pm 8.5	50.5 \pm 9.9*
2)	BMI	18.7 \pm 2.7	20.7 \pm 3.6*
3)	Hemoglobin (gms%)	11.3 \pm 2.2	11.9 \pm 1.8*
4)	CD4 (cell/mm ³)	243.3 \pm 173.8	355.7 \pm 225.2*
5)	CD4 (%)	14.7 \pm 9.7	18.3 \pm 9.2*

* denotes P value <0.05

Table VIII : Mean nutrient intake based on dietary assessment

S.No.	Nutrients	HIV+ves supplemented with 'Indiamix' (Mean ± S.D.)	
		Treated TB (n=144)	Without TB (n=422)
1)	Calories (kcal)	2054.0 ± 665.7	2073.7 ± 664.6
2)	Carbohydrates	359.0 ± 122.3	352.0 ± 121.2
3)	Protein (gms)	67.0 ± 32.1	67.0 ± 32.1
4)	Fat (gms)	38.8 ± 30.9	44.0 ± 26.2

and socioeconomically matched controls. This cohort will be followed for one year to assess changes in anthropometric, immunological and hematological parameters after nutritional supplementation.

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Drug susceptibility testing of *M. tuberculosis* using nitrate reductase assay

Background:

Drug susceptibility testing for *M. tuberculosis* using conventional methods is time-consuming or expensive as is the newer BACTEC method. With the increasing prevalence of TB and MDR-TB in the HIV-infected and non-infected patients, the felt need of the hour is an alternative for the traditional susceptibility testing methods. A rapid and inexpensive method has been reported recently. The test is based on the ability of *M.tuberculosis* to reduce nitrate to nitrite, which is routinely used for the biochemical identification of mycobacteria. The reduction is detected by using specific reagents, which produce a colour change.

Aims:

1. To use Nitrate Reductase Assay (NRA) as a novel inexpensive and rapid method of drug susceptibility testing of first line drugs and ofloxacin.
2. To compare the method with BACTEC radiometric method and conventional Proportional Sensitivity Test (PST) method on Lowenstein-Jensen (LJ) medium.

Methods:

A panel of clinical isolates of *M. tuberculosis*, (25 susceptible and 75 drug-resistant) along with H37Rv will be tested. All strains were taken from the collection of strains available from all over India will be used. Strains will be sub-cultured and inoculated into various media, i.e. LJ with drugs (PST), LJ with potassium nitrate and drugs (NRA) and into the BACTEC 12 B medium for susceptibility testing. Appropriate controls will be used with all testing procedures.

Conventional Susceptibility Testing:

PST method standard LJ medium with antibiotics in critical concentrations will be used.

NRA Susceptibility Testing:

In this method the strains will be inoculated using the above method in LJ slopes containing

1000 µg/ml potassium nitrate and drugs in similar concentrations as in PST.

BACTEC Method:

The 12 B medium will be inoculated using the standard BACTEC radiometric method for drug susceptibility testing.

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Bactericidal activity of PA 824, a nitroimidazopyran in various combinations with standard anti TB drugs against static culture of *M. tuberculosis*

Background:

PA 824, 5 nitroimidazole, a promising series of nitroimidazopyran, has been identified for the treatment of TB. This drug was found to show potent bactericidal activity against *M. tuberculosis* including MDR strains *in vitro* as well as *in vivo* in animal models (mice and guineapigs). It has comparable activity to isoniazid and isoniazid with combination of rifampicin, in the continuation phase of treatment tested in murine model. It was also shown that PA 824 (P) had activity under microaerophilic/anaerobic conditions thereby suggesting its potential sterilizing activity. This compound works by a novel mechanism inhibiting the protein and lipid synthesis. However, its activity in combination

with other anti- TB drugs has not been fully evaluated, especially with potent sterilizing drug such as pyrazinamide. To study the activity of Z *in vitro*, it requires acidic medium as established earlier our laboratory.

Aim:

To study the bactericidal activity of PA 824 in various combinations with standard anti TB drugs against static culture of *M. tuberculosis*.

Method:

M. tuberculosis H37Rv is grown in Middlebrook 7H9 medium at a pH 5.9 for approximately 30 days as a static culture. Later, these cultures in turn will be exposed to the identified drugs, either alone or in combinations with other anti-TB drugs as follows.

1. No drug 2. P1 (3 µg/ml) 3. P2 (12.5 µg/ml) 4. H 5. R 6. Z 7. M 8. HR 9. HRZ 10. HRP1 11. HRZM 12. HRZP1 13. HRZMP1 14. RZM 15. RZMP1 16. RZMP2

M. tuberculosis H37Rv is grown to attain a non replicative phase 2 (NRP2) stage. The results will be interpreted based on the ability of reduction in colony forming unit (cfu) after exposure to the drug in combination with H, R and Z.

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