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Analysis of drug susceptibility in *Mycobacterium tuberculosis* isolated from Thiruvananthapuram using Alamar Blue assay

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Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* and the control of the disease is hampered by widespread emergence of drug resistance in this pathogen. An early information on drug susceptibility would greatly facilitate an effective treatment of TB. Seventy-eight isolates of *M. tuberculosis* were obtained from TB patients from Thiruvananthapuram over a period of about 18 months. Resistance and susceptibility of these isolates to four frontline drugs were assayed using Alamar Blue, an oxidation–reduction dye. Thirty-six per cent of the isolates were susceptible to all the four drugs used, 21.8% were resistant to isoniazid, 8.9% to ethambutol and 2.6% to rifampicin. None was found resistant to streptomycin alone. Multidrug resistance (resistance to at least rifampicin and isoniazid) was found in 7.7% of the isolates. The remaining ones were resistant to combinations of two or more of the drugs. Alamar Blue-based assay promises to be an economical and fast method to determine drug susceptibility and resistance of *M. tuberculosis* to aid effective drug therapy.

AMONG the world's adult population, tuberculosis (TB) is the foremost cause of death from a single infectious agent[1]. In 1993 the World Health Organization declared TB as a global health emergency[2] and estimated that about three million people die of TB every year in the world, with about one-sixth of the deaths occurring in India. Human Immunodeficiency Viral (HIV) infection has been shown to increase the risk of developing tuberculosis. TB used to be completely curable by anti-TB drugs. Due to a variety of reasons[3], however, strains resistant to one or more of these drugs have emerged all over the world. In fact, multidrug-resistant (MDR) TB is the major hurdle in the TB control programmes. Global surveillance of drug resistance has been proposed as a means of augmenting databases of drug-resistant *Mycobacterium tuberculosis* isolates to help the development of future programme policy recommendations[4]. The widely followed Directly Observed Therapy, Short-

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course (DOTS) had its origin on studies carried out in Tamil Nadu, and several studies have been carried out in India on various aspects of TB. Kerala, though proclaimed to have the best healthcare system in the country, has scant data on TB. In the absence of any published data from Kerala, we initiated studies on prevalence of the disease and drug resistance, primarily focusing on Thiruvananthapuram district.

Treatment of TB without the benefit of information on susceptibility to drugs increases the risk of treatment failure and the spread of drug-resistant strains. This underscores the urgent need to determine the susceptibility pattern of *M. tuberculosis* rapidly for a more rational prescription of anti-TB drugs. Proportion and Minimal Inhibitory Concentration (MIC) methods are normally employed to assay drug susceptibility in *M. tuberculosis*. Although relatively inexpensive, the intensive labour involved at various steps and the long duration (3–4 weeks) required to obtain results make these methods cumbersome and inconvenient for routine analysis. Newer systems such as radiometric BACTEC 460 TB (Becton Dickinson, USA), which uses ^{14}C-labelled palmitic acid-containing liquid media with automated detection of $^{14}CO_2$ released[5], fluorescence-based BACTEC 9000 MB[6], fluorescence quenching-based Mycobacterium Growth Indicator Tube (MGIT) (ref. 7), luciferase *in vivo* expression technology[8], DNA probes[9] and hybridization protection assay[10] requires highly sophisticated and expensive equipment which cannot be afforded by most of the clinical laboratories in our country.

Alamar Blue, an oxidation–reduction dye, is an indicator of cellular viability and growth; the blue oxidized form turns pink upon reduction[11]. Yajko *et al.*[12] used this assay to find out the MICs of four frontline anti-TB drugs on 50 clinical isolates of *M. tuberculosis*. Collins and Franzblau[13] screened 30 antimicrobial agents against *M. tuberculosis* and *M. avium* using a microplate Alamar Blue assay and, Palomino and Portaels[14] have tested drug susceptibility of 94 isolates of *M. tuberculosis*.

In this paper we report the results of a preliminary study carried out in Thiruvananthapuram using Alamar Blue to assay drug susceptibility and resistance in *M. tuberculosis* isolated from persons diagnosed to have pulmonary TB. Early morning sputum samples were collected from smear-positive (based on microscopic observations made at the hospitals) patients attending the Chest Diseases Unit of the Taluk Hospital, Neyyattinkara and the TB Sanatorium, Pulayanarkotta, Thiruvananthapuram. The samples were processed by modified Petroff's method and bacteria were grown on Lowenstein–Jensen solid medium following standard techniques[15]. The resultant colonies were checked for their morphology and acid-fast staining property. The identity of the isolates was confirmed by standard biochemical tests–catalase activity at pH 7.0 at 68°C (negative), niacin production (positive) and nitrate reduction (positive). PCR of the isolates was done as a part of our attempt to standardize rapid identification of *M. tuberculosis*. All the isolates tested were positive for *devR* sequence[16].

Seventy-eight of the local isolates of *M. tuberculosis* were analysed for their susceptibility to rifampicin (RMP), isoniazid (INH), ethambutol (EMB) and streptomycin (SM). Stock solutions of antibiotics (Sigma, St. Louis, USA) were prepared in water, filter-sterilized, aliquoted and stored at – 20°C until used, except for RMP which was freshly prepared in dimethyl formamide. The critical concentrations were adapted from Rastogi[17]. Half and double the critical concentrations were also used for titration. The stocks were thawed on ice and were added to 450 µl of TB broth (HiMedia, Mumbai) in 1.5 ml sterile disposable microcentrifuge tubes to achieve 0.5X, 1X and 2X critical concentrations. The final concentrations were: RMP, 0.5, 1 and 2 µg/ml; INH, 0.1, 0.2 and 0.4 µg/ml; EMB, 2.5, 5 and 10 µg/ml; and SM, 1, 2 and 4 µg/ml. Three control tubes that did not have any drug were also set-up simultaneously. The microplate-based assay employed by the other authors was not attempted in our laboratory, even though it was convenient, as we felt that the risk involved in handling, as well as potential cross contamination in such open cultures were higher.

Colonies from 4-week-old subcultures were transferred to tubes containing 0.85% saline, thoroughly vortex mixed and the suspensions were allowed to stand for five minutes. Fifty microlitres of the supernatants were inoculated into all the tubes, and the tubes were mixed well and incubated at 37°C without shaking. On the 7th day, 25 µl of Alamar Blue solution (Accumed International, Ohio, USA) was added to the first control tube. If the colour changed from blue to pink, the dye was added to all the tubes and observed for a maximum of six hours. If the control did not change colour, all the tubes were incubated for another two days and the dye was added to the second control tube. If the colour change was not observed in the third control tube even on the eleventh day, the experiment was abandoned and repeated with a fresh culture (in our hands, colour developed in most cases within 1 to 2 h of addition of the dye on the 7th day itself). A pink colour in at least the first two tubes (0.5X and 1X critical concentrations of the drugs) was interpreted as resistance to that particular drug as the bacteria were viable *at* the critical concentration. Blue colour in the last two tubes (1X and 2X critical concentrations of the drugs), even if the first one was pink (as the drug concentration of 0.5X in this tube was too low to kill the bacteria) was considered sensitive as the drug killed all the bacteria *at* the critical concentration. *M. tuberculosis* strain H37Rv was used as control.

We have conducted proportion method analysis with H37Rv and three clinical isolates for a comparative study. H37Rv showed concordance in both the assays, as it did not grow on the medium containing any of the drugs and also did not alter the blue colour of Alamar Blue in any of

the tubes with drugs. In the case of clinical isolates, the results from both assays were found to be in full agreement with each other. Thus, for the limited number of samples tested, both the assays produced the same result. Of the 78 isolates analysed by Alamar Blue assay, 28 (36%) were sensitive to all the four drugs. Twenty-six (33.3%) were resistant to any one of the drugs – 17 (21.8%) to INH; 7 (8.9%) to EMB; 2 (2.6%) to RMP and none to SM. MDR (resistance to at least INH and RMP) was observed in 6 (7.7%) of the isolates. The remaining isolates had resistance to different combinations of INH, RMP, EMB and SM.

Yajko *et al.*[12] compared the susceptibility of *M. tuberculosis* to four frontline anti-TB drugs using Alamar Blue and proportion methods, and found 97% agreement between the two. Franzblau *et al.*[18] used microplate-based Alamar Blue assay to determine the MIC of frontline drugs of 34 Peruvian clinical isolates of *M. tuberculosis*. They have compared the results with those obtained from BACTEC 460 system and found 93.6% correlation between the two. Palomino and Portaels[14] have also compared the results obtained from Alamar Blue assay with those from the proportion method and found 97.1% overall agreement.

Drug resistance is a global phenomenon. In the 1960s and 1970s, single and MDR TB was low. In 1976, high levels of drug resistance were observed among Asian and Hispanic patients (20.7 and 15.0%, respectively)[19]. In a four-year long study conducted at Rawalpindi, 53% isolates from patients were found to be resistant to one of the frontline drugs[20]. Among the resistant isolates, 26.33% were resistant to INH, 24% to RMP, 28% to SM and 23.33% to EMB, with or without resistance to other drugs. MDR was found in 13.66%. A recent study conducted in Southern Mexico reported an overall rate of 28.4% resistance and 10.8% of MDR TB[21].

Information on the prevalence of TB and the extent of drug resistance in *M. tuberculosis* on a national basis is not available in India today. Most of the data come from studies conducted at the centres at Chennai (Tuberculosis Research Centre, TRC), Bangalore (National Tuberculosis Institute, NTI), Agra (JALMA) and some isolated studies conducted at medical colleges and hospitals. Available data show dramatic variation in drug resistance in different parts of the country. Surveys conducted at North Arcot district, Tamil Nadu and Pondicherry Union Territory revealed that the former had an initial resistance of 25% to one or more drugs and the latter 13% (ref. 22). In a study conducted at Jaipur (Rajasthan), resistance to one or more drugs was observed in about 20% of the patients[23]. A study conducted at Bombay Hospital Institute of Medical Sciences, Mumbai, showed that amongst the 521 sputum positive cases, 15% were resistant to INH, 66.8% to RMP, 8.4% to EMB, 53.6% to SM, 72.2% to pyrazinamide, 39.2% to cycloserine, 25.1% to kanamycin and 65.3% to ethionamide[24]. In a study conducted at L.T.M. Medical College, Mumbai, of the 100 patients tested for drug-resistant bacilli, 11 had MDR[25]. In our study we found that six (7.7%) patients harboured MDR bacilli and of them two patients had no history of anti-TB-treatment, indicating that MDR is spreading through primary infection in the community. This indeed is an alarming situation, although the proportion of MDR strains is relatively low compared to that in some other parts of the country. The lack of a uniform study and authentic database on drug resistance in India has been fortunately recognized and consequently a quality-controlled, systematic study monitored by TRC, Chennai and NTI, Bangalore is being planned[26].

Detection of drug susceptibility and resistance of *M. tuberculosis* is of paramount importance for the successful treatment of TB and for healthcare planning for the future. Belated availability of information on drug susceptibility would often make it clinically irrelevant. In our country there are only very few places where routine drug susceptibility tests of *M. tuberculosis* are performed and these centres may not be accessible to the patients. The conventional methods are not amenable to routine use at ordinary clinical laboratories. Alamar Blue-based method, on the contrary, does not require heavy financial commitment, with the cost of the dye for assaying a sample against four drugs coming to less than forty rupees and is easy to perform. It is faster and easier than conventional proportion and MIC methods, and is economical and equally fast when compared to the new hi-tech methods. The assay does not call for costly special equipment (though specific, prescribed precautions for handling *M. tuberculosis* have to be strictly met) or specialized training for performing the assay. Alamar Blue assay could be adapted to suit the laboratories in TB centres and even private clinics, making it an ideal system for use in developing countries. Advocating the widespread use of this technique definitely requires more thorough studies to compare the Alamar Blue assay with conventional techniques, though early results in our hands have been encouraging. Studies from other centres are required to fill this lacuna. We feel that in addition to comparing the two techniques, it is more important to determine the critical concentration for each drug that correlates with clinical resistance. Studies from several laboratories in India should come out with a quality controlled, standardized methodology for the routine use of this technique.

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A simple thermodynamic model for seasonal variation of monsoon rainfall

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Monsoons occur in the tropics on account of complex interaction between the continents, the ocean and the atmosphere and hence no simple model of the monsoon has been proposed so far. In this paper, a simple diagnostic model of the monsoon has been proposed based on the constraints imposed by energy and the moisture balance in a vertical column of the tropical atmosphere. The model demonstrates that the monthly mean rainfall depends upon evaporation, the net energy available in the atmosphere and the vertical stability of the atmosphere. The model simulates the seasonal variation of rainfall in India, Africa and South America remarkably well without explicitly invoking the concept of land–sea contrast in temperature. The model is useful to understand the impact of deforestation, greenhouse gases and aerosols on monsoon rainfall.

THE understanding and prediction of seasonal variation of the rainfall in the tropics has been a major goal in tropical meteorology. During the past twenty-five years, complex General Circulation Models (GCMs) of the atmosphere have been used to simulate the seasonal variation of precipitation in the tropics[1]. Although these models exploited the ability of the computer to perform millions of computations per second, they have not been very successful in simulating accurately the seasonal variation of precipitation during the monsoon[2]. This could be on account of poor representation of processes such as rainfall, cloud–radiation interaction, evaporation from vegetation and flow around mountains. On account of the inherent complexity of a GCM, it is difficult to identify the factors responsible for the poor simulation of monsoon rainfall by a GCM. Ironically, these complex models constructed by human beings are as inscrutible as nature. Hence a simple diagnostic model of the monsoon is necessary to identify the factors that govern the seasonal variation of rainfall. We discuss in this paper a simple diagnostic model based on the energy and moisture balance of the tropical atmosphere.

In the tropics, horizontal gradients of temperature and moisture are weak and hence their contribution to the energy balance can be neglected when compared to the contribution of the terms such as radiation, latent heat release or vertical gradient of temperature. In the tropics,

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