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RIFAMPICIN RESISTANCE PROFILE OF *MYCOBACTERIUM TUBERCULOSIS* ISOLATED FROM HUMAN PATIENTS

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Abstract: The recently increased tubercular incidence in certain parts of the world compels the need to explore its scientific reasons. For this purpose a total 172 clinical isolates of *Mycobacterium tuberculosis* were collected from TB diagnosed patients. The culture sensitivity test was performed over Lowenstein Jensen (LJ) medium supplemented with rifampicin at optimum concentration of 40 ug/mL. Resistant strains were 21.50%, while 78.50% were sensitive. Five further rifampicin levels were prepared to determine the highest possible resistance of *M. tuberculosis*. All of the 37 rifampicin resistant strains were resistant at 1st and 2nd drug levels, while 15 (40.5%) strains were inhibited at 3rd level (120 ug/mL), 13 (35.1%) at 4th level (160 ug/mL), 7 (18.9%) at 5th level (200 ug/mL) and 2 (5.4%) at higher than the 5th level (200+ ug/mL). These concentrations exceed the therapeutic index 6.5 ± 3.5ug/mL and therefore, cannot be maintained in the plasma of tubercular patients in actual clinical practice. Thus, it is suggested to replaced rifampicin by some other chemotherapeutic agent, modify its combination or find some other effective procedure to stop mortality and morbidity of terminally ill patients of Multidrug Resistant (MDR) and Extensively Drug Resistant (XDR) tuberculosis.

***Keywords*:** *M. tuberculosis*, rifampicin susceptibility, multidrug resistance

Introduction

Tuberculosis has been reported to be the major cause of death in adults among infectious diseases, being responsible for almost three million deaths annually in the world [1] that makes it the biggest killer after HIV. Almost 38% of the global TB burden is in the South-East Asian region, where annually 750,000 deaths occur due to TB, or 1500 everyday, or one every minute [2]. TB continues to challenge since antiquity. Rifampicin, which belongs to a group of macrocyclic antibiotics is frequently prescribed for tubercular treatment [3]. However, recent increase in the incidence of tuberculosis (TB) in certain parts of the world and the emergence of multi-drug resistant (MDR) strains, has urged the need for its rapid diagnosis and treatment. About 95% of TB cases occur in developing countries [4] and the disease has remained endemic for many decades [5]. Some physiological conditions and constant mutations may convert susceptible to resistant *M. tuberculosis* [6]. The mortalities because of this resistance have significantly decreased [7] due to confirmation of strains [8], application of molecular diagnostic techniques [9], investigation of transmission [10] and identification of the exogenous reinfection [11].

Rifampicin is derived from the soil mold *Streptomyces mediterranei* [12] and having broader antimicrobial activity [13]. Because resistant strains rapidly emerge during therapy, it is never given as a single agent in the treatment of active tuberculosis. It blocks transcription by interacting with the β subunit of bacterial DNA dependent RNA polymerase and inhibits RNA synthesis by suppressing the initiation step [14]. An alteration in the affinity or decreased permeability may produce resistance against rifampicin [15]. Rifampicin is moderately absorbed and distributed to all fluids and organs. The metabolites and parent drug are

eliminated via bile or urine [3].

In the present work, we have studied pulmonary and extra-pulmonary TB with primary and acquired rifampicin resistance. The major objective of this investigation was to study the resistance pattern of *M. tuberculosis* against rifampicin and explain certain therapeutical issues to design more effective therapy plan.

Materials and Methods

The study was conducted at Pakistan Medical Research Centre, King Edward Medical College, Lahore, National Institute of Biotechnology & Genetic Engineering (Faisalabad) and Department of Microbiology, Quaid-i-Azam University (Islamabad). Rifampicin was obtained from the Schazoo Laboratories (Pvt.), Lahore. A total 172 pulmonary and extra pulmonary tuberculosis diagnosed (AFB positive) patients were selected from five different local sources. They included 41 (23.8%) from Mayo Hospital-Outdoor, 110 (64%) from Mayo Hospital-Indoor, 14 (8.1%) from Jinnah Hospital, 6 (3.5%) from DOTS (Directly Observed Treatment Strategy, WHO) programmes and 1 (0.6%) from WAPDA Hospital, Lahore. The patients of all age groups were selected, regardless of their age, gender and previous therapeutic profile. The samples comprised 70.9% (122) males and 29.1% (50) females out of a total of 172 patients. Three different types of samples i.e. sputum, bronchial washing and pus were collected from all patients. The specimens were collected in screw-caped bottles.

Processing of Specimens

The liquefied sputum, pus or bronchial washings were centrifuged for 15 min and decontaminated with NaOH solution (40 g/L, 4% w/v). The organisms were sedimented and small quantity of distilled water added. The supernatant was discarded to obtain the residual concentrated specimen of *M. tuberculosis*. The concentrated residues of sputum, pus or bronchial washing were used for primary culture. Micro-Pippetman apparatus (Gilson, France) was used to take samples and poured in the center of LJ (Lowenstein Jensen) medium slides, which were moved up and down to spread the sample homogenously over the medium. This primary culture was kept in an incubator at 35-37^{o}C for 4 weeks. The growth of *M. tuberculosis* thus obtained was further used for sensitivity testing.

Preparation of Lowenstein Jensen (LJ) Medium

Lowenstein Jensen medium was prepared by the method described by Nazir *et al.* [16] and used for culturing *M. tuberculosis*. Approximately, 15 mL of medium was poured into sterilized 25 mL Bijoux Bottle (McCartney vials), secured with sterilized silver cap and kept at 85^{o}C in slanting position for 45 min. The medium was solidified/ hardened and kept at 115^{o}C for 10 min, cooled, labeled and stored at 2-8 ^{o}C.

Susceptibility testing of Mycobacterium tuberculosis

The drug sensitivity testing was performed within 1-2 weeks after obtaining the growth of *M. tuberculosis*. The sensitivity was evaluated against pyrazinamide by drug proportion method [17]. The patient's samples were processed in batches of 10-15 specimens. There were three control and two drug containing LJ medium slides.

Preparation of dilutions of M. tuberculosis

Approximately 1.0 mg/mL wet bacilli were estimated to vary between $\geq 10^6$ and 10^8 CFU. A representative sample of growth was taken from primary culture and placed into McCartney vials containing 1.0 mL of distilled water and 5 glass beads. This was homogenized by vigorous stirring by Vertex Mixer for 1-3 min and kept in safety cabinet. The opacity of the suspension was adjusted by the addition of sterile distilled water to that of a MacFarland standard No.5 (alternately a standard suspension of 1.0 mg/mL of BCG can be used). Five serial 10-fold dilutions of inoculums were

prepared i.e. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} in tubes labeled as 1, 2, 3 and 4, respectively. Tube No. 3 (dilution inoculum 10^{-3} and tube No. 5 (dilution inoculum 10^{-5}) were used for sensitivity testing.

The Bijoux bottles were inspected weekly for appearance of growth. When the growth was evident on LJ medium, colony morphology was noted. One culture bottle was taken and exposed to daylight for one hour and re-incubated. On the following day, it was examined for pigmentation. The cultures with no growth were discarded after 8 weeks of incubation. The presence and amount of growth was recorded on control and drug inoculated media. The results were interpreted for resistance on the basis of percentage of colonies on drug media in comparison to the growth on drug-free medium. The strains showing susceptibility were again incubated and examined after 6 weeks before declaring as sensitive. The growth pattern, number of colonies and contamination were checked carefully on weekly basis (Table 1).

Table 1. The number colonies and respective resistance of *M. tuberculosis*.

Codes	Stand for	Remarks
10	10 colonies	Might be resistant.
25	25 colonies	Mild resistant
50	50 colonies	Resistant
1+	More than 100 colonies	Severe Resistant
2+	More than 200 colonies	Highly resistant
3+	More than 300 colonies	Very highly resistant

Results and Discussion

A total 172 clinical isolates of sputum 146 (84.9%), pus 18 (10.5%) and bronchial washings 8 (4.7%) were collected from tuberculosis diagnosed patients. These comprised 145 (84.3%) pulmonary and 27 (15.7%) extra-pulmonary tubercular patients. These findings are in conformity with Bitar *et al.* [18] who reported 88.9% (n=607) pulmonary cases, 5.7% (n=39) extrapulmonary cases and 5.4% (n=37) cases of unknown site of disease.

Gender Distribution

There were 122 (70.9%) males and 50 (29.1%) females out of a total 172 patients. Gender comparison depicts greater percentage of male than female TB patients. Uplekar *et al* [19] have also reported a 70% excess of male patients over the female patients globally each year. The reasons for this gender difference are unclear as yet. Similar results have been reported by Haq *et al* [20] (68.3% male and 31.7% female tuberculosis patients). WHO/ IUALTD [21] has reported 67.2% male tuberculosis patients. These findings compare well with those of Bitar *et al.* [18] who have reported 70.2% male patients (ages ranging from 1 to 91 years, median = 35 years).

Susceptibility of Rifampicin

There were 37 cases (21.5%) of resistance and 135 cases (78.5%) of strain sensitivity to rifampicin. Out of the 37 (21.5%) rifampicin-resistant isolates (out of total 172) of *M. tuberculosis,* 1 (2.7%) strain had 20 colonies, 3 (8.1%) had 30 colonies, 6 (16.2%) had 50 colonies, 26 (70.3%) had 100 colonies and 1 (2.7%) had 200 colonies (Fig. 1). According to WHO/IUALTD records [21] also, there is 25.1%. rifampicin resistance in India and 39.3% in Estonia, and our data on rifampicin resistance are also supported by the works of Herendra and Shah [22], Rizwan *et al.* [23] and Miah *et al.* [17].

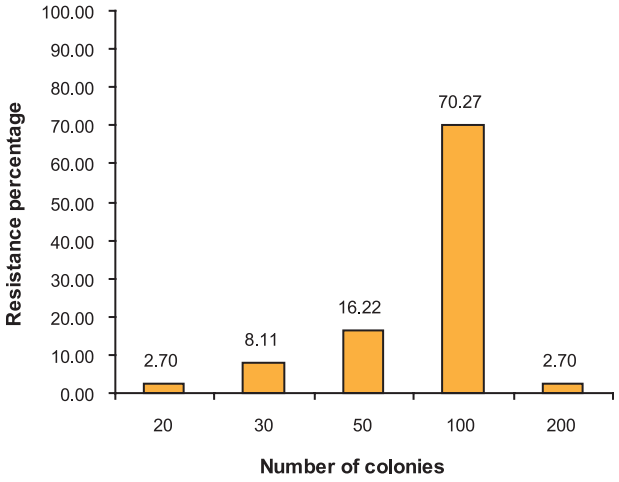

Figure 1. Comparison of resistance percentage and quantity of growth of indigenous *M. tuberculosis* in rifampicin incorporated Lowenstein Jensen media.

Maximum Therapeutical Resistance of *M. tuberculosis*

In terms of therapeutical and pharmacological comprehension, Cmax, therapeutic index, standard dose, maximum regimens and unwanted effects were studied and 5 rifampicin levels were prepared to determine the highest possible resistance of *M. tuberculosis*. All of the 37 (100%) strains were found resistant at 1st (40 µg/mL) and 2nd (80 µg/mL) rifampicin levels, while 15 (40.5%) strains were inhibited at 3rd level (120 µg/mL), 13 (35.1%) strains inhibited at 4th level (160 µg/mL), 7 (18.9%) strains inhibited at 5th level (200 µg/mL) and 2 (5.4%) strains inhibited at higher than 5th level (>200 µg/mL) (Table 2). These concentrations exceeded the therapeutic range of rifampicin [14]. The concentrations of rifampicin incorporated in LJ medium and that maintained in the patient's plasma are different. The critical determinant of therapeutic effectiveness of rifampicin is the functional state of host defense mechanisms [14]. The standard combinations of rifampicin used for miscellaneous time frames [13] are the exact evidence of effectiveness of lower plasma concentration in combination therapy than in the standard MIC of mono drug therapy. The unwanted effects of rifampicin are nausea, vomiting, rashes, hepatitis, GI upset, flu like syndrome, fever, jaundice [3] red-orange colored urine and tears [14]. Quantitative difference of dose required in individual and combination therapy has been studied by Stephen [6], who has reported that resistant tuberculosis poses a significant threat to human health and is important in understanding how the resistance has emerged. It may help to reverse the upward trend. Treatment with internationally approved regimens has resulted in high cure rates, without the emergence of resistance. These regimens are effective in preventing the emergence of resistance because of inhibition of the development of spontaneous resistance due to mutation. These results are supported by earlier finding of Centre of Disease Control and Prevention [24], which reported rifampicin's MIC for *M. tuberculosis* in the range of 1-50 ug/mL. Karin *et al.* [25] Grimm [26], Rizwan *et al.* [23] and Canada Communicable Disease [27] reported 40 ug/mL as optimum concentration for declaration of sensitive or resistant *M. tuberculosis*. Richard *et al.* [3] reported C_{max} (maximum plasma concentration) of 6 ± 3.5 ug/mL, MIC 0.005-0.2 ug/mL, 98% protein binding, 0.9 L/Kg volume of distribution (Vd) and standard therapeutic dose of 600 mg with maximum regimen of 1100 mg per day. Our findings are also in line with Bertram [15] who reported 1.0 ug/mL MIC, 5-7 ug/mL maximum plasma concentration and 600 mg daily dose. Venkatesan [28] reported peak serum concentration for adults as 7-9 ug/mL after a single 600 mg oral dose and for children (6 to 58 months of age) approx 11 ug/mL after a dose of 10 mg per Kg body weight, Vd 1.6 L/Kg and 89% protein binding. Because of high lipid-solubility, rfampicin may reach and kill susceptible intracellular as well as extracellular bacteria. These results are also consistent with Juan [29] who reported rifampicin's daily adult dosage of 10–20 mg/Kg and 8–12 mg/Kg for children with 600 mg maximum daily regimen. Praharaj *et al.* [30] also reported *in vitro* resistance at >64 ug/mL.

Table 2. Level of resistance (in % age) of rifampicin resistant *M. tuberculosis.*

RFP level in LJ Media	Rifampicin ug/ mL	No. of MTB Strains	Percent Resistance	Valid Percent	Cumulative Percent
1	40	37	100.00		
2	80	37	100.00		
3	120	15	40.54	40.54	40.54
4	160	13	35.13	35.13	75.67
5	200	7	18.91	18.91	94.59
5+	200+	2	5.40	5.40	100.00
	Total	37	100.00	100.00	

From the data presented here it is evident that seriously sick and hospitalized tubercular patients need special attention. Prevalence of pulmonary tuberculosis is greater than that of extra-pulmonary tuberculosis. Furthermore, rifampicin resistance covers approximately 1/5th of total strains. Maximum resistance was seen at 1st and 2nd drug levels. While minimum resistance was noted

at higher than 5th level. Any of the determined concentrations is not possible to maintain in the plasma of tuberculosis patient in actual clinical practice, because of the limitations of optimum therapeutic range of 6.5 ± 3.5 ug/mL. Thus, it is suggested that rifampicin should be replaced by some other chemotherapeutical agent, or modify its combinations or find some alternate effective procedure which may help stop the mortality and morbidity of terminally ill patients of Multidrug Resistant (MDR) and Extensively Drug Resistant (XDR) tuberculosis.

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