PYRAZINAMIDE RESISTANCE OF *MYCOBACTERIUM TUBERCULOSIS STRAINS, ISOLATED* FROM HUMAN PATIENTS

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Summary

Pyrazinamide resistance emerged as a major cause of failure of anti-tubercular therapy. The risk of infection with resistant mycobacterium is increasing posing a threat to control and eradicate this disease. The main reasons of emergence of resistance included erratic drug ingestion, monotherapy, omission of one or more prescribed agents and suboptimal doses. The resistance could be acquired during the treatment with other antitubercular drugs. Freely available anti TB drugs in the markets of third world countries also lead to self medication and improper regimen which eventually proliferate the susceptible to resistant strains. The recent increase in the incidence of this disease in certain parts of the world and emergence of resistant has urged the need of searching its scientific reasons. To assess the scientific and accurate pyrazinamide resistance we have collected 172 clinical isolates of *Mycobacterium tuberculosis* (*M. tuberculosis*) from TB diagnosed patients from six different sources. The specimens comprised of 84.9% sputum, 10.5% pus and 4.7% bronchial washings with 84.30% pulmonary and 15.69% extra-pulmonary tuberculosis. Forty Severn (47) *M. tuberculosis* strains were confirmed as resistant by culturing over Lowenstein Jensen (LJ) medium supplemented with pyrazinamide at optimum concentration of 100ug/ml. These resistant strains were then further studied to determine their highest level of resistances (in % age). Five pyrazinamide concentrations levels were prepared to determine the highest possible resistance of *Mycobacterium tuberculosis*. Non pyrazinamide resistant strains inhibited at 1st and 2nd levels, while 13 (27.66%) strains inhibited at 3rd level of 300g/ml, which is not possible to maintain in the plasma of tuberculosis patients, because of its exceeding the optimum therapeutic range of 9-12ug/ml (1), 19ug/ml (2), 30-50ug/ml (3), 37-40ug/ml (4). In addition to this it was found that 12 (25.53%) strains inhibited at 4th level (400 ug/ml), 15 (31.91%) inhibited at 5th level (500ug/ml) and 7 (14.89%) inhibited at higher than 5th level (>500 ug/ml). These levels also exceed the therapeutic index and therefore can not be recommended in actual clinical practice. Thus it is suggested to replace pyrazinamide with some other chemotherapeutic agent, modify its combination or find some other effective procedure to stop mortality and morbidity of terminally ill patients of resistant *M. tuberculosis*.

Keywords: *Mycobacterium tuberculosis*, pyrazinamide susceptibility and resistance

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Introduction

Pyrazinamide resistant *M. tuberculosis* strains are a serious threat to successful tuberculosis control programmes (5). Some physiological conditions and constant mutations induce a hypermutable state and making multiple resistances (6) that may affects the molecular mechanism of resistance needed for intracellular survival of *M. tuberculosis*. Pyrazinamide resistant organisms become more virulent and required to be treated and prevented to transmit in community. It has also emerged as a major cause of failure of anti-tubercular therapy and may be acquired during the treatment with certain other antitubercular drugs i.e. rifampicin, pyrazinamide. The genetic mutations and certain physiological conditions produce the multiple resistances that may affects the molecular profile needed for intracellular survival of *M. tuberculosis*. The recent increase in the incidence of this disease in certain parts of the world and emergence of isoniazid resistant strains has urged the need of searching the scientific reasons.

Pyrazinamide is a synthetic, bactericidal agent used in combination with rifampicin, isoniazid and ethambutol. It kills the actively dividing organisms in the acidic environment of lysosomes as well as in macrophages (3) and must be enzymatically hydrolyzed to pyrazinoic acid (the active form of pyrazinamide). The objective of this investigation was to study the resistance pattern of *M. tuberculosis* and explain certain therapeutical issues to design most effective therapy plan.

Materials and Methods

Pyrazinamide was obtained from the Schazoo Laboratories (Pvt.) limited and all other chemicals obtained from market and research laboratories. A total number of 172 pulmonary and extrapulmonary tuberculosis diagnosed (AFB positive) patients were selected from five different sources (Table 1). The patients of all age groups were selected, regardless of their age, gender and previous therapeutic profile. The samples comprised of 70.9% (122) males and 29.1% (50) females out of a total of 172 patients (Table 2). Three different types of samples i.e. sputum, bronchial washing and pus were collected from all patients (Fig.1). The specimens were collected in screw caped bottles.

Processing of Specimens

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

Table 1. source and number of samples of *M. tuberculosis* strains

	Source	Frequency	Percent	Valid %age	Cumulative %age
1	Mayo Hospital Outdoor	41	23.8	23.8	95.9
2	Mayo Hospital Indoor	110	64.0	64.0	64.0
3	Jinnah Hospital	14	8.1	8.1	72.1
4	DOTS	6	3.5	3.5	99.4
5	WAPDA Hospital	1	0.6	0.6	100.0
	Total	172	100.0	100.0	

Table 2. Gender wise patient distribution

	Frequency	Percent	Valid Percent	Cumulative Percent
Females	50	29.1	29.1	29.1
Males	122	70.9	70.9	100.0
Total	172	100.0	100.0	

Preparation of Lowenstein Jensen (LJ) Medium

Lowenstein Jensen medium was prepared by the method described in detail by Taha *et al.*, (18) and used for culturing the *M. tuberculosis*. Approximately 15ml of medium was poured into sterilized 25cc McCartney vials. Closed with sterilized silver cap and kept at 85^{o} C in slanting position for 45 minutes. The medium was solidified/ hardened and kept at 115^{o}C for 10 minutes, 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 growth of *M. tuberculosis*. The sensitivity was evaluated against pyrazinamide by drug proportion method (7). 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 1mg (wet weight) bacilli/ ml were estimated to vary between $\geq 10^{6}$ and 10^{8} CFU. A representative sample of growth (containing minimally 50 colonies) were taken from primary culture and placed into McCartney vial containing 1ml of distilled water and 5 glass beads. This was homogenized by stirring by Vertex Mixer for 1-3 minutes and left in safety cabinet. The opacity of the suspension was adjusted by addition of sterile distilled water to that of a Mac Farland standard No.5 (alternately a standard suspension of 1.0 mg/ ml of BCG was used). Four serial 10 fold dilutions of inoculums were prepared i.e. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} in tubes labeled as 1, 2, 3 and 4, respectively. The tube number 3 of dilution inoculum of 10^{-3} and tube number 5 of dilution inoculum (10^{-5}) were used to culture for sensitivity testing.

Recording the Results

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 day light for one hour and re-incubated. On following day examined for pigmentation. The cultures with no growth were discarded after 8 weeks of incubation. The presence and amount of growth in term of number of colonies on control and drug inoculated medium were recorded (Table 4). 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.

Results and Discussion:

Out of 172 samples 41(23.8%) outdoor of Mayo Hospital, 110(64%) Indoor of Mayo Hospital, 14(8.1%) Jinnah Hospital, 6(3.5%) WAPDA Hospital, Lahore, Pakistan (Table 1). The specimens comprised of 146(84.9%) sputum, 18(10.5%) pus and 8(4.7%) bronchial washings (Fig.1) with 145(84.30%) pulmonary and 27(15.69%) extra-pulmonary specimens (Fig.2). These findings are in conformity with Bitar *e. al.,* (8) who have reported that majority of cases 89% (n=607) were of pulmonary TB, 6%(n=39) presenting extra-pulmonary tuberculosis and 5%(n=37) cases for whom site of disease was unknown.

Gender Distribution:

There were 122(70.9%) males and 50 (29.1%) females out of total 172 clinical isolates (Table 2). Gender comparison depicts greater percentage of male than females. These findings have been substantiated by Uplekar *et al* (9), who reported a seventy percent (70%) excess of males over females globally each year. The reasons for this difference are unclear as yet. The findings of this study are also consistent with the findings of Haq *et al.,* (10) who have reported 68% males and 32% females tuberculosis patients. Our findings are also in conformity with WHO/ IUALTD (7) that reported 67% of male tuberculosis patients. These findings are in agreement with Bitar *et el* (8), who have reported 70% males with a M:F sex ratio of 2:8. Their ages ranged from 1 to 91 years (median = 35 years).

Susceptibility of Pyrazinamide

Data showed the pyrazinamide susceptibility profile as 47 (27.3%) resistant and 125 (72.7%) sensitive strains (Fig. 3). On basis of number of colonies, resistance was found as 1 (2.13%) strain had 30 colonies, 4 (8.51%) had 50 colonies, 39 (82.98%) had 100 colonies, 3 (6.38%) had 200 colonies out of 47 (27.32%) pyrazinamide resistant isolates of a total of 172 *M. tuberculosis* (Fig.4). These finding are in conformity with Bitar *et al.,* (8), who reported 25% of resistant to pyrazinamide (one-third of cases with missing information). The findings of this study are in line with the work of Joel, (1), who reported the resistance prevalence and molecular level comprehension of resistance against pyrazinamide. These finding are substantiated by Rizwan *et al.,* (11), who reported 29% resistance to against pyrazinamide. Primary and acquired resistance was 19% and 39% for pyrazinamide which was statistically significant different.

Level of Pyrazinamide Resistance and Its Therapeutical Interpretation

The resistance against pyrazinamide interpreted therapeutically to study the pharmacological suitability. Standard dose, maximum regimens, Cmax, therapeutic index, and unwanted effects were studied to understand the therapeutic comprehension. *M. tuberculosis* strains were inoculated over the medium containing five different pyrazinamide levels. All of the 47 (100%) strains were found resistant at 1st (100µg/ml) and 2nd levels (200µg/ml). 13 (27.66%) strains were resistant upto 3rd level (300µg/ml), 12 (25.53%) strains upto 4th level (400µg/ml), 15 (31.91%) strains upto 5th level (500µg/ml) and 7 (14.89%) strains higher than 5th level (>500 µg/ml) (Table 5). These concentrations incorporated in LJ medium exceed the therapeutic range of 9-12ug/ml (1), 19ug/ml (2), 30-50ug/ml (3) and 37-40ug/ml (12), therefore can't be maintained in plasma of tuberculosis patients in actual clinical practice. Exceeding this maximally permitted plasma concentration may introduce sever health hazards. Maintenance of aforesaid plasma concentration is therefore rejected to use in actual chemotherapeutical practice. During the pyrazinamide toxicity the important adverse effect introduced by pyrazinamide are the nausea, hyperuricemia, rashes, joint ache, gout (rare) (12), fever, anorexia, malaise, and hepatitis with or without hepatic jaundice, and death can occur (13). 25.53% resistant *M. tuberculosis* were inhibited at 4th pyrazinamide level 400ug/ml, 31.91% resistant stains inhibited at 5th pyrazinamide level 500ug/ml and 14.89% pyrazinamide resistant stains inhibited at higher than 5th pyrazinamide level 500+ug/ml. The respective plasma concentration at 5th level of 500ug/ml and higher than 5th level of 500+ug/ml can not be maintained in tuberculosis patient without the risk of sever patient's health hazards. Therefore regimens are rejected to use in actual chemotherapeutical practice. The findings of this study are in line with the work of Richard *et al.,* (4), who reported 18-23ug/ml minimum inhibitory concentration, 37-40ug/ml maximum plasma concentration, 500mg standard therapeutical dose with maximum regimen of 800mg. Our findings are consistent by the work of Praharaj *et al.,* (12), who reported the maximum plasma concentration (Cmax) 40ug/ml, critical drug concentration that incorporated in LJ media to consider as border line for declaration of sensitive or resistant bacteria 100ug/ml, standard dose 500mg and maximally permitted dose 800mg. Our finding are in line with the work of Bertram *et al.,* (3) who reported the minimum inhibitory concentration 100ug/ml, maximum plasma concentration 30-50ug/ml and standard dose of 25mg/kg/day. These findings are in conformity with Juan, (13), who reported the usual pyrazinamide dosage 25mg/kg/day with a maximum of 2g per day. Pyrazinamide administered orally and having the bactericidal effect. Its daily dosage for children 20–30 and for adult is 1.5g <50kg 2.0g (51–74kg) 2.5g >75kg. Thrice weekly dose for adult is 2g <50kg 2.5g (51–74kg) 3.0g >75kgl. The action of pyrazinamide is fundamental on slow-multiplying and intercellular bacilli and has a sterilizing effect. It is hepatoxic and interferes with the metabolism of uric acid by increasing its level. The Volum of distribution of pyrazinamide is 0.57 to 0.74 L per kg (16). These findigns are in line with Ellard, (14), who reported widely distribution of pyrazinamide to most fluids and tissues, including liver, lungs, kidneys, and bile. Pyrazinamide has excellent penetration into CSF, ranging from 87 to 105% of the corresponding serum concentration. These findigns are in agreement with Stamatakis *et al.,* (15), who reported the plasma protein binding 10-20%), time to peak serum concentration 1-2 hours and peak serum concentration 19 ug/ml after a single dose of 14 mg/kg and 39 ug/ml after a single dose of 27 mg/kg. The findings of this study are in line with the work of Praharaj *et al.,* (14), who reported the final concentration (100ug/ml) incorporated in LJ media to consider as border line to declare the

sensitive or resistant *M. tuberculosis* was very much clearly higher than the 100ug/ml. Study by Joel *et al.,* (1), also reveal a similar findings of 12.5 minimum inhibitory concentration, 9-12ug/ml maximum plasma concentration following a regimen of 500mg per day. Quantitative difference of doses required in individual and combination therapy has been studied by Stephen (6) who has reported that drug-resistance tuberculosis poses a significant threat to human health and is important to understand how the resistance emerges if we are to reverse the upward trend. Treatment with internationally approved regimens has resulted in a very high cure rates, without the emergence of resistance. These regimens are effective in preventing the emergence of resistance because of inhibiting the development of spontaneous resistance due to mutation.

Most seriously ill tubercular patients were generally seen in hospitals, requiring special attention. They might also contribute contagious nosocomial tubercular infection. The epidemiological data has pointed out five time higher prevalence of pulmonary tuberculosis than extra-pulmonary tuberculosis. Approximately ¼ of the total resistance observed against pyrazinamide. Maximum resistance observed at 1st & 2nd pyrazinamide levels and minimum at above than 5th level. The final concentrations incorporated in media have exceeded the therapeutic index, therefore can not be recommended in actual clinical practice. Thus it is suggested to replace pyrazinamide with some other chemotherapeutic agent, modify its combination or find some other effective procedure to stop mortality and morbidity of terminally ill patients of resistant *M. tuberculosis.*

Table 3: Ingredients of Lowenstein Jensen media.

	Ingredients	12000	1800
1	Magnesium sulfate (MgSO4.7H2O)	0.15 g	0.27 g
2	Magnesium citrate (Mg - Citrate)	0.375 g	0.674 g
3	L- Asparagines	2.20 g	4.05 g
4	Potassium Dihydrogen Sulfate (KH2PO4)	1.5 g	2.7 g
5	Glycerin	7.5 g	13.5 ml
6	Malachite green	12.5 g	22.5 g
7	Distilled Water	375 g	675 g
8	Eggs	625 ml	1125 ml

Table 4. 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

Table 5. Level of resistance (in % age) of pyrazinamide resistant *M. tuberculosis*

PZA Levels in LJ Media	Pyrazinamide ug/ ml	No of MTB Strains	Percent Resistance	Valid Percent	Cumulative Percent
1	100	47	100		
2	200	47	100		
3	300	13	27.660	27.660	27.660
4	400	12	25.532	25.532	53.191
5	500	15	31.915	31.915	85.106
5+	500+	7	14.894	14.894	100
	Total	47	100	100	

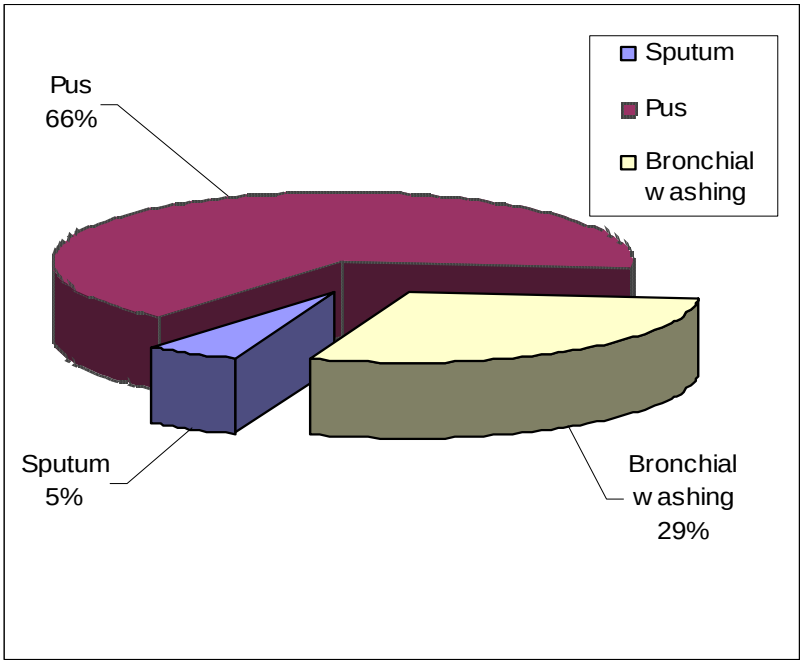

Fig.1. The number and types of samples collected from Tuberculosis (AFB) positive patients.

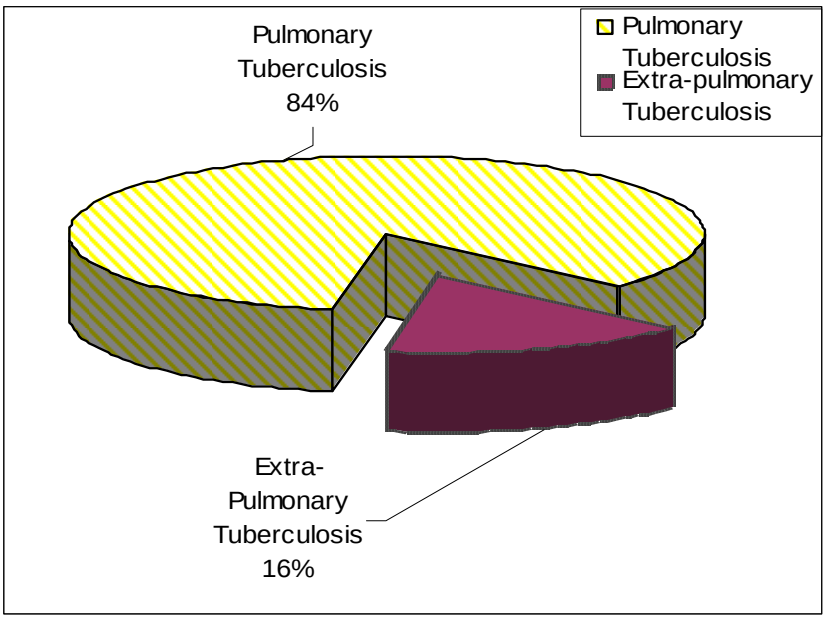

Fig. 2. Distribution of the pulmonary and extra-pulmonary tuberculosis specimens:

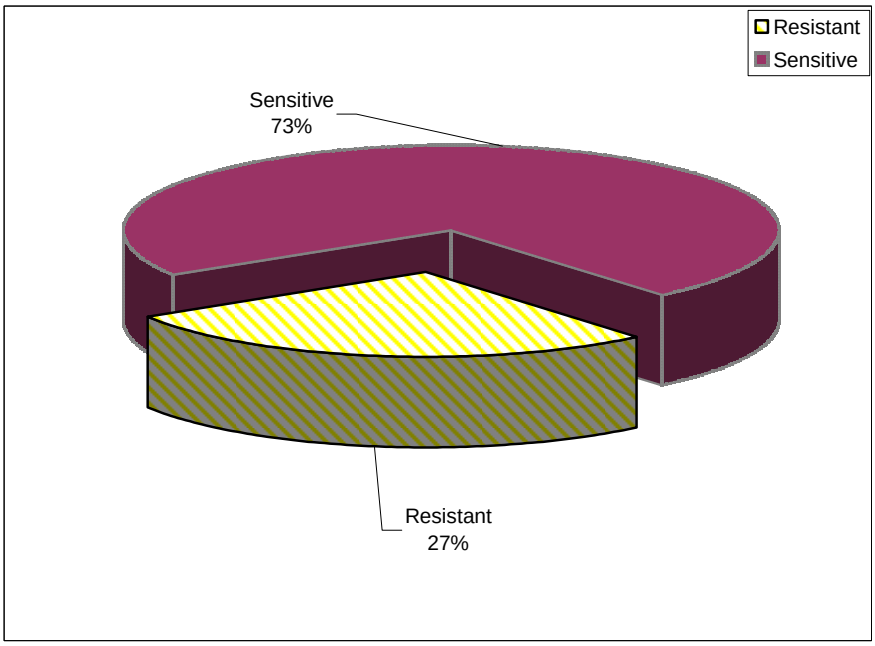

Fig. 3. Pyrazinamide resistance pattern of indigenous *M. tuberculosis* strains collected from primary culture of TB diagnosed (AFB positive) patients.

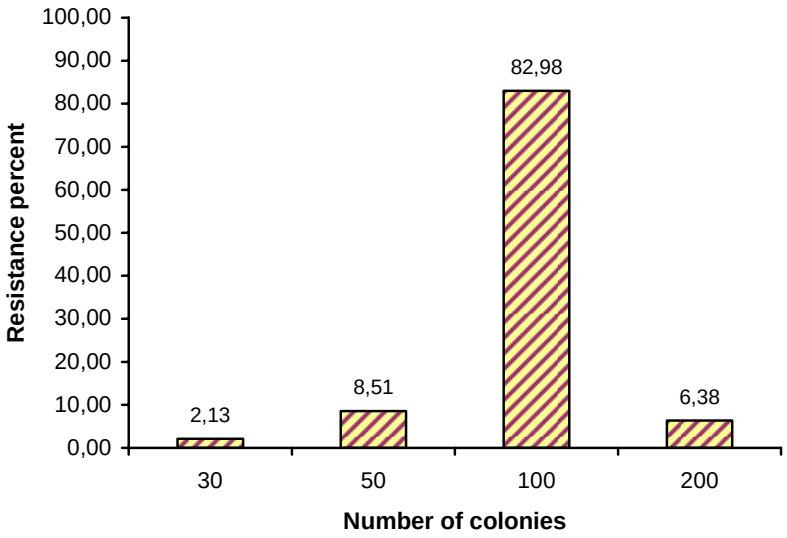

Fig. 4. Comparison of resistance pattern (percentage) and quantity of growth of indigenous *M. tuberculosis* in pyrazinamide incorporated Lowenstein Jensen media

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