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Full Length Research Paper

Isoniazid resistance of *Mycobacterium tuberculosis* isolated from human patients

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Isoniazid resistant *Mycobacterium tuberculosis* strains are a serious threat for successful tuberculosis control programs. Therefore, present study was aimed to figure out the pattern and level of resistance of *M. tuberculosis* against isoniazid. A total of 172 specimens of sputum, pus and bronchial washings; 70.9% males and 29.1% females with 84.30% pulmonary and 15.69% extrapulmonary tuberculosis were collected from six different sources. The inoculums were prepared using 0.5 Mac Farland turbidity standards. Five concentrations of isoniazid were used in Lowenstein Jensen (LJ) medium that is 3, 6, 9, 12 and + 12 µg/ml for sensitivity testing. Data showed 25 (14.5%) resistant and 147 (85.5%) sensitive *M. tuberculosis* strains. The growth was not inhibited at first (3 µg/ml) and second (6 µg/ml) drug levels, while 36% isolates inhibited at third level (9 µg/ml), 28% at forth level (12 µg/ml) and 24% at fifth level (10 µg/ml) and 12% at higher than fifth Level (> 12 ug/ml). These incorporated drug concentrations are higher than therapeutic index and not recommended in actual clinical practice. It is thus obvious to explore some other effective chemotherapeutic agents, modify combinations or figure out more effective procedures to stop morbidity and mortality due to isoniazide resistance of *M. tuberculosis*.

Key words: Isoniazid, *mycobacterium tuberculosis,* resistance, Lowenstein Jensen medium.

INTRODUCTION

The emergence of isoniazid resistance is alarming, the problem of great importance concern to public health. Certain clinical, biological and epidemiological risk factors are constantly contributing to develop resistance. Primarily, it is induced because of the inaccurate treatment protocols, substandard socio-economic condition and poor therapeutical surveillance (Koh et al., 2005). It may also be caused by genetic mutations, certain physiological conditions and therapeutical errors that eventually proliferate the resistant strains (Nolan and Goldberg, 2002). The recent increase in the incidence of this disease and emergence of resistance has urged the need to search for some more effective procedure to control mortalities due to multidrug resistance (MDR) tuberculosis.

The major factors of emergence of isoniazid resistance are erratic drug ingestion, monotherapy, omission of one or more prescribed agents and suboptimal doses. The half of all new TB cases are in 6 Asian countries – Bangladesh, India, Pakistan, Indonesia and Philippine and multidrug resistance (isoniazid and rifampicin resistance with or without resistance against other anti-TB drugs) present virtually in all 109 countries (WHO, 2005). In most developing countries, the disease has always been endemic (Herendra and Shah, 1998) but in industrialized countries the development of resistance, suppressed immunity and contributed the disease's resurgence (Barnes et al., 1991). The anti TB drugs are freely available in market that leads to self medication and improper regimen. However, an optimal regimen and

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duration for this treatment remains a matter of some controversy. Here, we tied to share our experience in a case of pulmonary and extra-pulmonary TB with primary and acquired isoniazid resistance. The major objective of our investigation was to study the resistance pattern of *M. tuberculosis* against isoniazid and explain certain therapeutical issues to decide clinical credibitily of isoniazid in tuberculosis treatment programs.

MATERIALS AND METHODS

This study was conducted at Pakistan Medical Research Centre (PMRC), King Edward Medical College, Lahore, National Institute of Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan and Department of Microbiology, Quaid-i-Azam University Islamabad, Pakistan. Pure chemical of isoniazid was obtained from the Schazoo Laboratories (Pvt.), Lahore.

Collection and processing of samples

A total number of 172 pulmonary and extra-pulmonary tuberculosis diagnosed (AFB positive) patients were selected from six different sources. The patients of all age groups were selected, regardless of their age, gender and previous therapeutic profile. The sputum, pus and bronchial washings were collected, labeled and stored, separately. All the samples were centrifuged decontaminated and processed as described by Taha et al. (2010). These concentrated residues of sputum, pus and bronchial washings were used for primary culture.

Preparation and inoculation of Lowenstein Jensen (LJ) medium

Lowenstein Jensen medium was prepared by the method described by Nazir et al. (Uplekar et al., 2001) and used for primary culturing of the processed samples. A 15 ml of medium was poured into sterilized 25 ml McCartney vials and closed with sterilized silver caps. The medium was autoclaved at 115°C under 15 lb/inch^2 pressures for 20 min. Then it was solidified in slanting position, cooled, labeled and stored at 2 to 8°C. The LJ medium slants were inoculated with processed samples in class-II safety cabinet (Telstar, Spain). The inoculated slants were kept in incubator at 37°C for 4 weeks. The growth of *M. tuberculosis* thus obtained was identified though acid fast staining and further used for sensitivity testing.

Preparation of inoculums of *Mycobacterium tuberculosis*

The over seeding of a drug containing medium was prevented by standardization of inoculums that mitigate the possibility of miss judgment of considering the resistance to a susceptible strain. Approximately 1 mg wet weight bacilli/ml was estimated to vary between 106 and 108 CFU. The representative samples of growth containing minimally 50 colonies were taken from primary culture and placed into McCartney vials containing 1 ml of distilled water and 5 glass beads. The mixtures were homogenized by vigorous stirring by Vortex Mixer (Eyela, Japan) for 1 to 3 min and left in class-II safety cabinet (Telstar, Spain). The opacity of the suspensions was adjusted by the addition of sterile distilled water to that of a 0.5 Mac Farland turbidity standard. Four serial 10 fold dilutions of inoculums were prepared that is 10 to 1, 10 to 2, 10 to 3 and 10 to 4 in sterilized test tubes and labeled as 1, 2, 3 and 4, respectively. The tube No. 3 of dilution inoculums of 10 to 3 and tube No. 5 of dilution inoculums 10 to 5 were used to culture for sensitivity testing.

Susceptibility testing of *M. tuberculosis*

The drug sensitivity testing was performed within 1 to 2 weeks after obtaining primary growth of *M. tuberculosis*. The sensitivity was evaluated against isoniazid by drug proportion method as recommended by world health organization (WHO) and International Union against Tuberculosis and lung diseases (Richard et al., 2006). The patient's samples were processed in batches of 10 to 15 specimens on drug containing LJ medium and drug free control LJ medium. Five different concentration of isoniazid were used in LJ medium that is 3, 6, 9 and 12 µg/ml. Each LJ medium slant was inoculated by using the above inoculums prepared from primary cultures of *M. tuberculosis* and incubated at 37°C for 4 weeks.

Recording and interpretation of 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 exposed to day light for 1 h and re-incubated to be examined for pigmentation on the following day. 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 recorded. The results were interpreted for resistance on the basis of percentage of colonies on drug containing media in comparison to the growth on drug free medium. The strains showing susceptibility were again incubated and examined at 6 weeks before declaring as sensitive. The growth pattern, number of colonies and contamination were checked carefully on weekly basis.

RESULTS

A total number of 172 samples were collected from six different local sources; 41 (23.8%) outdoor, Mayo Hospital, 110(64%) indoor, 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 with 145 (84.30%) pulmonary and 27 (15.69%) extra-pulmonary specimens. There were 122 (70.9%) males and 50 (29.1%) females out of total 172 clinical isolates (Table 2).

Data showed the isoniazid susceptibility profile as 25 (14.5%) resistant and 147 (85.5%) sensitive strains. On basis of number of colonies resistance was found as; 30 and 50 colonies shown two different strains, 100 colonies by 21 (84%) strains and 200 colonies by resting 2 (8%) strain out of 25 (14.53%) isoniazid resistant isolates of a total 172 *M. tuberculosis* (Table 3).

Furthermore *M. tuberculosis* strains were inoculated over the LJ medium incorporated with five different isoniazid levels. All of the 25 (100%) strains were founded resistant at first (0.2 µg/ml) and second levels (3 µg/ml). 9 (36%) strains were resistant up to third level (6 µg/ml), 7 (28%) strains upto forth level (9 µg/ml), 6 (24%) strains up to fifth level (12 µg/ml) and 3 (12%) strains

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

S/N	Source	Frequency	Percentage (%)	Valid (%)	Cumulative (%)
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.

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

Table 3. Comparison of resistance percentage over quantity of growth of indigenous *Mycobacterium TB* in isoniazid incorporated Lowenstein Jensen media.

			Valid	Cumulative
Number of colonies	Frequency	Percentage (%)	Percentage (%)	Percentage (%)
30	1	4	4	4
50	1	4	4	8
100	21	84	84	92
200	2	8	8	100
Total	25	100	100	

higher than fifth level (>12 µg/ml) (Table 4).

DISCUSSION

These findings are in conformity with Bitar et al. (2001) 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 comparison depicts greater percentage of male than females. These findings have been substantiated by Uplekar et al. (2001) who reported a 70% excess of males over females globally each year. The reasons for this difference are unclear as yet. These findings are also in consistent with Haq et al. (2002) who reported 68% male and 32% female tuberculosis patients. Our findings are in conformity with WHO/IUALTD (2000) that reported 67% of male tuberculosis patients. These findings are in agreement with Bitar et el. (2001) who have reported 70% males with a Male: Famale sex ratio of 2:8.

Our findings are supported the work of WHO/IUALTD (2000), who reported the 19.4% isoniazid resistance in Russian Federation, 11.3 % resistance in china and 15.4% resistance in India. Study by Canada Communicable Disease Report (2004), reveals similar finding of 9.3% most common type of drug resistance against isoniazid. The possible reason of difference of results may lie in the facts of the differences in living standards, health facilities, over all hygienic condition and socio-economic privileges. These findings are in agreement with Herendra and Shah (1998), who reported 20 to 30% isoniazid resistance. These findings are in consistent with Miah et al. (2000) who reported 15.8% isoniazid resistance and 5% MDR in Bangladesh. Our finding are in line with Sumathi and Srivastava (2004), who reported 11 (14.7%) isolates were seen resistant to isoniazid by all the three methods - proportion method by agar dilution on Middlebrook 7H11 agar, proportion method using the conventional Lowenstein-Jensen (L-J) medium and E test strip method. Findings of the study under discussion substantiated by Rizwan et al. (2003), who reported the 26% isoniazid resistance and 16% MDR. Primary and acquired isoniazid resistance was 20 and 33% with statistically significant difference.

The findings regarding the level of isoniazid resistant

Table 4. Level of resistance (in % age) of isoniazid resistant *Mycobacterium TB.*

ISN Levels in LJ media	Isoniazid ug/ ml	MTB strains	Resistance (%)	Valid (%)	Cumulative (%)
1	0.2	25	100	0	0
2	3	25	100	0	0
3	6	9	36	36	36
4	9	7	28	28	64
5	12	6	24	24	88
5+	12+	3	12	12	100
	Total	25	100	100	

clinical isolate of *M. tuberculosis* are in line with the work of Jesudason et al. (2003), who reported the resistance of *M. tuberculosis* above than optimum concentration of 0.2 ug/ml. This work also supported by Victor et al. (1997), who reported *M. tuberculosis* resistance against isoniazid more than 0.2 ug/ml. our finding are also in line with Stephen (2002), who studied the quantitative difference of doses required in individual and combination therapy. He reported that isoniazid resistant 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 deve-lopment of spontaneous resistance due to mutation. The concentrations of isoniazid incorporated in LJ medium exceed the therapeutic range of 4 ug/ml Richard et al. (2006), therefore can not be maintained in plasma of tuberculosis patients in actual clinical practice. The maximum isoniazid regimen is 400 mg (Leon et al., 2004). If exceeded, unwanted effects including hypersen-sitivity, paresthenia, hepatic enzyme elevation, hepatitis and peripheral neuropathy, nausea, vomiting, epigastric distress, agranulocytosis, thrombocytopenia, fever, various rashes, dermatological and rheumatoid and lupoid syndromes may produce serious health hazards. The adverse effects are related to dosage and duration of administration. Paresthenia is the most common adverse effect, appears to be due to a relative pyridoxine deficiency. Which is corrected by pyridoxine (vitamin B6) supplementation and therefore particularly recommended to lactating women to intake vitamin supplementation? The findings of the study under discussion substantiated by Richard et al. (2006), who reported the standard MIC as 0.2 to 0.4 ug/ml, plasma concentration 4 ug/ml, maximally allowed plasma concentration 6 ug/ml, the standard therapeutical dose 300 mg with maximum regimen of 300 mg. These finding are substantiated by WHO/IUALTD (2000), who reported 58.33% resistance at first drug level 0.2 ug/ml, 8.33% resistance at second drug level 3 ug/ml and 33.33% resistance at fifth drug level 12 ug/ml. Our findings are in conformity with Joel et al. (2001), who reported 0.2 ug/ml MIC, 300 mg standard dose, maximum plasma concentration in slow acetylation 5.4 ± 2.0 ug/ml and in fast acetylation 7.1 ± 1.9 ug/ml. These findings are in consistent with Holdiness (1984), who reported the peak serum concentration 3 to 7 ug/mL (21.9 to 51 micromoles/L) after a single 300 mg oral dose. 0.57 to 0.76 L/ kg is the volume of distribution (Kergueris, 1983). 1 to 2 h peak serum concentration time (tmax) and 4 ug/ml peak plasma concentratioin (Cmax) (Venkatesan, 1989). Findings of study under discussion are substantiated by Juan (2006), who reported oral or IM administered of isoniazid with bactericidal effect. Its daily dosage for children is 5 to 10 mg/kg and for adult is 5 mg/kg. The maximum daily administrable regimen is 300 mg.

In conclusion, most seriously ill tubercular patients are generally seen in hospitals, the epidemiological data has pointed out five time higher prevalence of pulmonary tuberculosis than extra-pulmonary tuberculosis. The final concentrations incorporated in LJ medium exceeded the therapeutic index and can't be used in actual clinical practice. Therefore; we have to replace, modify or finding some other effective procedure to stop the mortality and morbidity due to isoniazid resistant *M. tuberculosis*.

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