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ETHAMBUTOL RESISTANCE OF INDIGENOUS *MYCOBACTERIUM TUBERCULOSIS* ISOLATED FROM HUMAN PATIENTS

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ABSTRACT

The present study was conducted to find out the ethambutol resistance pattern of indigenous isolates of *Mycobacterium tuberculosis* from Tuberculosis diagnosed human patients. A total of 172 specimens were collected from six different sources and comprised of 84.9% sputum, 10.5% pus and 4.7% bronchial washings. There were 70.9% males and 29.1% females with 84.30% pulmonary and 15.69% extra-pulmonary tuberculosis. The *Mycobacterium tuberculosis* isolates collected from primary culture were further studied to determine their pattern and level of resistance. The inoculums were prepared using 0.5 Mac Farland turbidity standards. Five different concentration of ethambutol were used in Lowenstein Jensen (LJ) medium i.e. 2µg/ml, 4µg/ml, 6µg/ml, 8µg/ml and 10µg/ml for sensitivity testing. Data showed 10 (5.8%) resistant and 162 (94.2%) sensitive *Mycobacterium tuberculosis* out of total 172 clinical isolates. The growth was not inhibited at 1st (2µg/ml) and 2nd (4µg/ml) drug levels, while growth of 50% isolates inhibited at 3rd level (6µg/ml), 30% inhibited at 4th level (8µg/ml) and 20% at 5th level (10µg/ml). The last three levels are above the therapeutic index and not recommended in actual clinical practice. It is thus conceivable to explore some other more effective chemotherapeutic agents, modify combinations or find more effective procedures to stop morbidity and mortality due to ethambutol resistant *Mycobacterium tuberculosis*.

Key words: Ethambutol; *Mycobacterium tuberculosis*; Resistance; Lowenstein Jensen medium; Human patients.

INTRODUCTION

Advancements in health care facilities offered a goal to eradicate tuberculosis (TB) by the end of the 20th century, but it reemerged because of the global resistance against anti-tubercular drugs. The 95% of TB cases occur in developing countries (9) and the disease remained endemic for many decades (7). Tuberculosis (TB) is the biggest killer of young adults in their economically most productive age. Nine million new cases of TB occur every year. Two million people died annually, that makes it the biggest killer after HIV. Poorly implemented directly observed treatment strategy (DOTS)

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gives rise to greater resistance against tubercle bacilli and prevalence of resistant *Mycobacteria* warrants through DOTS plus resistant TB control programs. Almost 38% of the global TB burden is in the South East Asian (SEA) region where annually 7, 50,000 deaths occur due to TB. Five of the 22 high burden countries are in the SEA Region (14).

Ethambutol act by inhibiting the Biosynthesis of arabinogalactan. The gene responsible for resistance against this drug is EmbCAB that produce the Arabinosyl transferase that allows continuation of arabinan biosynthesis (12). Arabinosyl transferase is an enzyme that is important for the synthesis of mycobacterial arabinogalactan cell wall. Resistance is not a serious problem if the drug is employed with other antitubercular agents. Ethambutol can be used in combination with pyrazinamide, isoniazid and rifampicin. The EmbCAB proteins are believed to be integral membrane proteins, consistent with their role in the synthesis of various arabinan-linkage motifs of the arabinogalactan and lipoarabinomannan. Planned and supervised therapy with judicious surgical interference is the only way to ensure cure for the patient and prevention of the spread of drug resistance (4). Ethambutol is a bacteriostatic drug and specific for most strains of *Mycobacterium tuberculosis* and *Mycobacterium kansasi*.

The objective of this investigation was to study the pattern and level of resistance of indigenous *Mycobacterium tuberculosis* isolated from tuberculosis diagnosed human patients against ethambutol which is the 1st line antitubercular drug.

MATERIALS AND METHODS

Collection and Processing of Samples

Pure chemical of ethambutol was obtained from the Schazoo Laboratories (Pvt.), Lahore. 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 at 1500 rpm for 15 minutes and decontaminated with NaOH 40g/ L (4%w/v) solution. The supernatant fluids were discarded and sediments were collected. 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.* (8) and used for primary culturing of the processed samples. A 15ml of medium was poured into sterilized 25mL McCartney vials and closed with sterilized silver caps. The medium was autoclaved at 115^{o}C under 15 lb/inch^{2} pressure for 20 minutes. Then it was solidified in slanting position, cooled, labeled and stored at 2-8^{o}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^{o}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 1mg wet weight bacilli/ml was estimated to vary between $\geq 10^{6}$ and 10^{8} CFU. The representative samples of growth containing minimally 50 colonies were taken from primary culture and placed into McCartney vials containing 1mL of distilled water and 5 glass beads. The mixtures were homogenized by vigorous stirring by Vortex Mixer (Eyela, Japan) for 1-3 minutes 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 i.e. 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} in sterilized test tubes and labeled as 1, 2, 3 and 4, respectively. The tube No. 3 of dilution inoculums of 10^{-3} and

tube No. 5 of dilution inoculums 10^{-5} were used to culture for sensitivity testing.

Susceptibility testing of *Mycobacterium tuberculosis*

The drug sensitivity testing was performed within 1-2 weeks after obtaining primary growth of *M. tuberculosis*. The sensitivity was evaluated against ethambutol by drug proportion method as recommended by WHO and International Union against Tuberculosis and Lung Diseases (15). The patient's samples were processed in batches of 10-15 specimens on drug containing LJ medium and drug free control LJ medium. Five different concentration of ethambutol were used in LJ medium i.e. 2µg/ml, 4µg/ml, 6µg/ml, 8µg/ml and 10µg/ml. Each LJ medium slant was inoculated by using the above inoculums prepared from primary cultures of *Mycobacterium 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 one hour 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 AND DISCUSSION

A total number of 172 samples were collected from six different local sources i.e. 41(23.8%) Outdoor, Mayo Hospital, 110(64%) Indoor, Mayo Hospital, 14(8.1%) Jinnah Hospital, 6(3.5%) WAPDA Hospital, Lahore, Pakistan. The specimens comprised of 146(84.9%) sputum, 18(10.5%) pus and 8(4.7%) bronchial washings with 145(84.30%) from pulmonary and 27(15.69%) extra-pulmonary tuberculosis cases. These findings are in conformity with Bitar *et al.* (2) who also reported that majority of cases 89% (n=607) were of pulmonary, 6%(n=39) presenting extra-pulmonary tuberculosis and 5%(n=37) cases for whom site of disease was unknown.

There were 122(70.9%) males and 50 (29.1%) females out of total 172 clinical isolates. Gender comparison depicts greater percentage of tuberculosis in males than females. These findings have been substantiated by Uplekar *et al.* (13) 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.* [3] who also reported 68% male and 32% female tuberculosis patients. Our findings are also in conformity with WHO/ IUALTD (15) which reported 67% of male tuberculosis patients.

The critical concentration of Ethambutol maintained in LJ media to consider as border line for declaration of resistant was 2µg/ml. The results of this study showed 10 (5.8%) resistant and 162 (94.2%) sensitive out of total 172 clinical isolates of indigenous *Mycobacterium tuberculosis*. Quantitatively out of 10 resistant isolates on LJ medium, 1 (10%) had 10 colonies and 9 (90%) had 100 colonies. Our findings are in line with the reports of WHO/ IUALTD, (15) which reported ethambutol resistance as 5.8% in Iran and 4.1% in china. These finding are also in conformity with Bitar *et al.* (2) who reported 4.4% ethambutol resistance. These results are similar to Khan *et al.* (5) who reported markedly increased resistance in Saudi Arabia during the last 5 years, except pyrazinamide and ethambutol showing resistance of 8% & 7%, respectively.

The indigenous *Mycobacterium tuberculosis* isolates were also inoculated in the medium containing five different ethambutol concentrations/levels. All of the 10 (100%) isolates were found resistant at 1st (2µg/ml) and 2nd (4µg/ml) levels. The 5 (50%) isolates were resistant up to 3rd level (6µg/ml), 3(30%) up to 4th level (8µg/ml), and 2 (20%) at higher than 5th

level (>10µg/ml) (Table 1). These concentrations incorporated in LJ medium exceed the therapeutic ranges of 3-5µg/ml (1), 2-5µg/ml (6) and 4-6µg/ml (11). These exceeding than the mentioned plasma concentrations may introduce toxicity in actual clinical practice. The most important adverse effects of ethambutol are optic neuritis (diminished visual acuity and loss of ability to discriminate red and green colors), blurred vision, and decreased urate excretion which may lead to gout (11). Additionally drug fever, abdominal pain, headache, dizziness and confusion may also occur. Discontinuation of the drug results in reversal of the toxic symptoms (6).

Table 1. Level of ethambutol resistance (% age) among indigenous *Mycobacterium tuberculosis*

Ethambutol Concentrations (µg/ ml)	No of resistant MTB Strains	Percentage Resistance
2	10	100
4	10	100
6	5	50
8	3	30
10	2	20
Total	10	100

The 3(30%) ethambutol resistant *Mycobacterium tuberculosis* isolates were inhibited at 4th level 8µg/ml and 2 (20%) inhibited at higher than 5th level (>10µg/ml). These concentrations can not be maintained without the risk of sever health hazards, therefore not recommended for use in actual chemotherapeutic practice. The findings of this study are in line with the work of WHO/ IUALTD (15) which reported 75% ethambutol resistance at 1st drug level, 12.5% at 2nd and 12.5% at 4th level. These finding are also in conformity with the work of Praharaj *et al.* (10) who reported the resistance of ethambutol above than the critical concentration of 2µg/ ml.

During this study, the most seriously ill tuberculosis patients were generally seen in hospitals, requiring special attention. They might also contribute to the spread of contagious nosocomial tubercular infection in the society. The epidemiological data has pointed out five time higher prevalence of pulmonary tuberculosis than extra-pulmonary tuberculosis. Approximately 1/16 of total *Mycobacterium tuberculosis* isolates were resistant to ethambutol. Maximum resistance was observed at 1st & 2nd drug levels and minimum at above than 5th level. The final concentrations incorporated in LJ medium have exceeded the therapeutic index, thus not recommended in actual clinical practice. Therefore we have to treat resistant patients by replacing and modifying the existing drugs or by exploring some other novel and more effective methods to stop the morbidity and mortality due to the ethambutol resistant *Mycobacterium tuberculosis*.

REFERENCES

1. Bertram, G.K. (2004). Basic and Clinical Pharmacology. McGraw Hill, New York.
2. Bitar, D.; Infuso, A.; Barboza, P.; Euro, T.B.; Heersma, H.; Kremer, K.; Soolingen, D.V.M.; Fauville, D.; Havelkova, M.; Prikazsky, V.; Lillebaeck, T.; Gutierrez, C.; Kubica, T.; Niemann, S.; Brum, L.; Iglesias, M.J.; Martin, C.; Samper, S.; Ghebremichael, S. (2001). Clustering of multi-drug resistant tuberculosis cases from nine European countries. Institut de Veille Sanitaire, Saint Maurice, France.
3. Haq, M.A.; Khan, S.R.; Saeed, S.U.; Iqbal, S.; Shabbir, R.; Magsi, J. (2002). Sensitivity Pattern of *Mycobacterium tuberculosis* at Lahore, Pakistan. *Annals King Edwards Med.Univ.* 8 (3), 190-193.
4. Herendra, T.; Shah J.R. (1998). Multidrug resistance pulmonary tuberculosis. *Ind. J. Tubercul.* 45, 114-117.
5. Khan, M.Y.; Kinsara, A.J.; Osoba, A.O. (2001). Increasing resistance of *Mycobacterium tuberculosis* to anti-TB drugs in Saudi Arabia. *Int. J. Antimicrob.* 17 (5), 415-418.
6. Leon, S.; Mutnick, H.A.; Souney, F.P.; Swanson, N.L. (2004). Comprehensive Pharmacy Review. West Camden Street, Baltimore, MD.
7. Maria, I.; Moura, F.; Caldas, P.C.S.; Said, A.; Martins, F.; Brito, C.R. (2004). Antimicrobial susceptibility determined by the E-test, Lowenstein Jensen proportion, and DNA sequencing methods among *Mycobacterium tuberculosis* isolates - discrepancies, preliminary results. *Mem. Inst. Oswald Cruz Rio de Janeiro*. 99: (1), 107-110.
8. Nazir, T.; Hameed, A.; Akhtar, M.S.; Shabbir, E.; Qureshi, J.A.; Malik, A.; Qaisar, M.N. (2008). Pyrazinamide resistance of *Mycobacterium tuberculosis strains isolated* from human patients. *Pharmacol. Online.* 3, 948-957.
9. Pablos, M.A.; Raviglione, M.C.; Laszlo, A.; Binkin, N.; Rieder, H.L.; Bustreo, F.; Cohn, D.L.; Lambregts, C.S.B.; Kim, S.J.; Chaulet, P.; Nunn, P. (1998). Global surveillance for anti-tuberculosis drug resistance. *Engl. J. Med.* 338, 1641-1649.

10. Praharaj, A.K.; Kalghatgi, A.T.; Varghese, S.J.; Nagendra, A. (2004). Incidence and drug susceptibility pattern of *Mycobacterium tuberculosis* in HIV infected patients. *Military J. Armed Forces Ind.* 60, 134-136.
11. Richard, D.H.; Mycek, J.M.; Harvey, R.A.; Champe, P.C. (2006). Lippincott's Illustrated reviews: Pharmacology. Baltimore.
12. Takayama, K.; Kilburn, J.O. (1989). Inhibition of synthesis of arabinogalactan by ethambutol in *Mycobacterium smegmatis*. *Antimicrob. Agent Chemother.* 33, 1493-1499.
13. Uplekar, M.W.; Rangan, S.; Weiss, M.G.; Ogden, J.; Borgdorff, M.W. (2001). Attention to gender issues in tuberculosis control. *Int. J. Tubercul. Lung Dis.* 5 (3), 220-224.
14. WHO (2004). World Heath Organization Surveillance of drug resistant tuberculosis in South East Asia. Report of an Inter country training course Bangalore, India.
15. WHO/IUALTD. (2000). Anti-tuberculosis drug resistance in the world report No. 2, prevalence and trends. Geneva. World Heath Organization, USA.