ORIGINAL ARTICLE

Extensively and Pre-Extensively Drug Resistant Tuberculosis in Clinical Isolates of Multi-Drug Resistant Tuberculosis Using Classical Second Line Drugs (Levofloxacin and Amikacin)

Irfan Ali Mirza[1], Farooq Ahmad Khan[2], Khursheed Ali Khan[3], Luqman Satti[4], Tahir Ghafoor[5] and Muhammad Fayyaz[5]

Abstract

Objective: To find out the frequency of Extensively Drug Resistant (XDR) and pre-XDR tuberculosis in clinical isolates of Multi-Drug Resistant (MDR) Tuberculosis (TB) by determining the susceptibilities against Levofloxacin and Amikacin (classical second line antituberculosis drugs).

Study Design: A descriptive cross-sectional study.

Place and Duration of Study: Microbiology Department, Armed Forces Institute of Pathology (AFIP), Rawalpindi, from September 2011 to August 2013.

Methodology: Amikacin (AK) and Levofloxacin (LEVO) were obtained in chemically pure form from Sigma (Taufkirchen, Germany). The breakpoint concentration used for AK was 1.0 μg/ml and for LEVO 2.0 μg/ml. Mycobacterial Growth Indicator Tube (MGIT) 960 system was used to carry out drug susceptibility testing as per recommended protocol.

Results: A total of 3 MDR-TB isolates (3%) turned out to be XDR-TB based upon simultaneous resistance to injectable second line antituberculosis drug AK and one of the fluoro-quinolones (LEVO). A total of 24 MDR-TB isolates (24%) were found to be pre-XDR based upon resistance to LEVO alone. Treatment status record of patients with XDR and pre-XDR-TB isolates revealed that majority of patients had received fluoroquinolones (FQs) during the course of treatment.

Conclusion: XDR-TB has started to emerge in MDR-TB isolates in our set up. The worrying sign is the high frequency of pre-XDR tuberculosis. Urgent steps need to be taken to stem the tide of pre-XDR-TB in our population. It is thus recommended to develop facilities to carry out drug susceptibility testing to monitor the status of pre-XDR and XDR-TB in our population.

Key Words: *Extensively drug resistant tuberculosis. Pre-extensively drug resistant tuberculosis. Multi drug resistant tuberculosis. Levofloxacin. Amikacin.*

INTRODUCTION

For thousands of years, humankind has been engaged in fight against *M. tuberculosis*. During all these years, the human beings could only rely on the efficiency of natural defenses of its own immune system, thus paying a toll of 50% mortality within 5 years after the onset of the disease.[1] During the two decades from the discovery of Streptomycin in 1943 to that of Rifampicin in 1963, there was significant turnaround in this battle as the disease turned from one of devastation to that of a fully curable one.[2]

Extensively Drug-resistant Tuberculosis (XDR-TB) is defined as TB caused by a Multi-Drug-Resistant (MDR) strain (i.e., resistant to at least Rifampicin and Isoniazid) that is also resistant to any Fluoroquinolone (FQ) and one of the second-line injectable drugs, such as Capreomycin, Kanamycin, or Amikacin (AK).[3] Pre-XDR TB is defined as MDR-TB strain that is resistant to either Fluoroquinolone or second line injectable drug but not both.[3] The emergence of drug-resistant strains of *M. tuberculosis* is significantly challenging the goal of elimination of TB in the 21st century. The estimated global number of MDR and XDR-TB cases among newly diagnosed patients with pulmonary TB in the year 2012 were in the range of 300,000 - 600,000. Only 28% of these estimated MDR/XDR-TB cases were notified. The incidence rate of TB in Pakistan was ranked fifth in the world and the estimated percentage of new TB cases with MDR-TB was reported to be 3.5%.[4]

Keeping in view the gravity of the disease, countries in the west are cognizant of the situation. In response to the alarming problem of MDR/XDR-TB, all 53 member states of the World Health Organization (WHO) European region have fully endorsed the consolidated action plan to prevent and combat MDR/XDR-TB in the WHO European region during 2011 - 2015 and its accompanying resolution at the 61st session of the WHO Regional Committee for Europe.[5]

[1] *Department of Microbiology, PNS Shifa, Karachi.*
[2] *Department of Microbiology, Rai Medical College, Sargodha.*
[3] *Department of Microbiology, Baqai Medical University, Karachi.*
[4] *Department of Microbiology, Combined Military Hospital, Quetta.*
[5] *Department of Microbiology, Armed Forces Institute of Pathology (AFIP), Rawalpindi.*

Correspondence: Dr. Irfan Ali Mirza, Consultant Microbiologist, PNS Shifa, Karachi.
E-mail: irfanmirza651@hotmail.com

Received: November 07, 2014; Accepted: March 28, 2015.

As only very few centres in Pakistan are carrying out drug susceptibility testing for *M. tuberculosis*, the objective of study was to find out the frequency of XDR and pre-XDR-TB in clinical isolates of MDR-TB in our set up by determining the susceptibilities against LEVO and AK (classical second line antituberculosis drug) by Mycobacterial Growth Indicator Tube (MGIT) 960 method.

METHODOLOGY

The descriptive, cross-sectional study was carried out at Microbiology Department, Armed Forces Institute of Pathology (AFIP), Rawalpindi, from September 2011 to August 2013. Prior permission was obtained from the institutional ethical review board for the study.

All MDR-TB isolates (resistant to Isoniazid and Rifampicin) recovered from different clinical specimens as part of routine drug susceptibility testing were subjected to susceptibility testing against two classical second line drugs, AK and LEVO. History of Anti-TB treatment received, especially FQs was retrieved from the clinical details mentioned on the Laboratory request form and recorded. Non MDR-TB clinical isolates were excluded from the study; similarly repeatedly cultured clinical isolates of the same patient's specimen were also excluded from study.

The Anti-TB, drugs AK and LEVO were obtained in chemically pure form from Sigma (Taufkirchen, Germany). AK disulfate salt, 710 μg/mg, Catalog Number A1774 with storage at 2 - 8°C manufactured by Sigma and LEVO (> 98% HPLC) Catalog Number, 28266 with storage at 2 - 8°C manufactured by Sigma were used. These drugs were dissolved in deionized water. The stock solutions of AK (84 μg/l and LEVO (84 μg/ml) were prepared in sterile water as per instructions provided in leaflets of respective drugs. These stock solutions were filtered through 0.22 μm pore size Millex-GS filter units (Millipore, Bedford, MA), aliquoted and stored at -70°C. The working solutions of AK and LEVO were diluted from the stock solution, aliquoted and frozen for future use.

The critical concentrations of AK and LEVO used for BACTEC MGIT 960 system were 1.0 μg/ml and 2.0 μg/ml.[6] Before subjecting the MDR-TB isolates to the test drugs fully susceptible and quality control strain, American Type Culture Collection (ATCC 27294) was subjected to the critical concentration of the drugs used. The drugs panel consisted of three MGIT tubes, one for growth control and two for second line drugs. Each 7 ml MGIT tube was checked for any contamination or turbidity and labelled properly. After mixing the growth supplement Oleic acid, Albumin, Dextrose and Catalase (OADC), 0.1 ml of each antibiotic stock solution was added in respective MGIT tubes. Quantity equivalent to 0.5 ml of culture proven MDR-TB sample was added to two MGIT tubes while 0.5 ml of 1:100 diluted sample was added to the control tube. After bar code scanning, all the inoculated tubes were entered in the instrument and incubated at a temperature of 37ºC. An un-inoculated MGIT tube was used as a negative control.

The MGIT 960 system fully supports the susceptibility testing of various combinations of Streptomycin, Isoniazid, Rifampicin and Ethambutol (SIRE) and Pyrazinamide (PZA) panels configured by the manufacturer, but second-line drug panels are not available. Testing of second-line drugs were registered in the MGIT 960 system as one of the SIRE panels and were manually entered for the drug identification on the printout of the results.[7] The MGIT 960 system indicates the conclusion of a process when the Growth Unit (GU) of the growth control reaches 400 and reports S for susceptible or R for resistant, as well as a GU value for each drug-containing MGIT tube on the printout. An isolate was interpreted to be susceptible when the GU of a drug-containing MGIT tube was equal to or less than 100 or as resistant when the GU was greater than 100. If an isolate was interpreted to be resistant, a smear was made and stained to prove the presence of Acid Fast Bacillus (AFB) with morphology compatible with that of *M. tuberculosis* and the absence of contaminants.

The statistical analysis of the data was done by the software Statistical Package for Social Sciences (SPSS) version 19. Descriptive statistics was calculated in terms of mean, Standard Deviation (SD), minimum, maximum and the range. Mean and SD was calculated for quantitative variables like age of patients. Frequency and percentages were calculated for susceptibility and resistance of MDR-TB isolates to AK and LEVO.

RESULTS

A total of 3 MDR-TB isolates (3%) turned out to be XDR-TB based upon simultaneous resistance to injectable second line antituberculosis drugs AK and LEVO (Table I). These three XDR-TB isolates were recovered from two males and one female, all of less than 40 years of age. Based upon resistance to only one second line drug (LEVO), a total of 24 MDR-TB isolates (24%) were found to be in the category of Pre-XDR (Table II). Pre-XDR isolates were recovered from 16 males and 8 females with a mean age of 34.08 ± 12.9 years (ranging from 15 to 63 years). Treatment status record of patients with XDR and pre-XDR-TB isolates revealed that majority of patients had received FQs during the course of treatment (Table III).

Table I: Percentage of extensively drug resistant - tuberculosis (XDR-TB) isolates.

Number of multi-drug resistant tuberculosis (MDR-TB) isolates	Amikacin (AK) (1.0 μg/ml) and Levofloxacin (LEVO) (2.0 μg/ml) resistant	
	Number	Percentage
100	3	3%

Table II: Percentage of pre-extensively drug resistant - tuberculosis (XDR-TB) isolates (resistant to Levofloxacin only).

Number of multi drug resistant tuberculosis (MDR-TB) isolates	Resistant to Levofloxacin (LEVO) (≤ 2.0 μg/ml)	Percentage resistant
100	24	24%

Table III: Treatment history profile of patients with XDR and pre-XDR isolates (n=27).

Status of Fluoroquinolone intake during treatment	Number of patients
Fluoroquinolones taken as part of treatment	15
Fluoroquinolones not taken as part of treatment	07
No history available	05

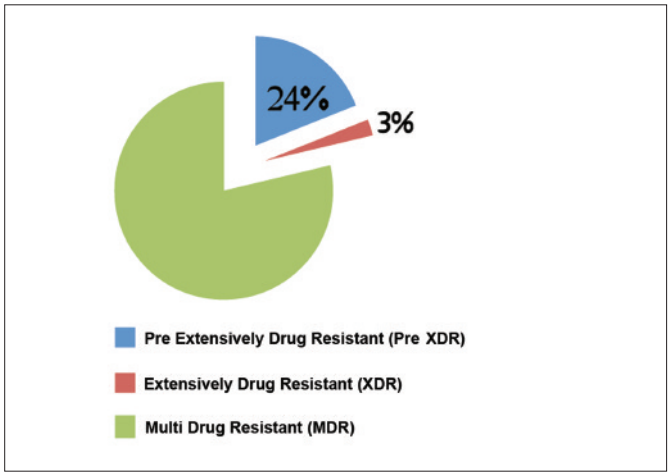

Figure 1: Distribution of XDR and Pre-XDR in (MDR) TB isolates (n=100).

DISCUSSION

With the increase in MDR-TB strains around the globe, there has been an urgent need to carry out drug susceptibility to first and second line anti-tuberculosis drugs. It is imperative that treatment of patients suffering from drug resistant TB should be carried out based on quick, reliable and quantitative measure of susceptibility testing. This endeavor is in fact the cornerstone for prevention of resistance in treatment of TB and a way forward for optimal exploitation of the available anti-tuberculosis drugs.

According to the WHO global report 2013, TB culture facility in Pakistan is possible in only seven laboratories accounting to 0.2 laboratory per 5 million population while in whole country, only four laboratories can perform drug susceptibilities accounting to only 0.1 laboratory per 5 million population. To add fuel to the fire, Pakistan also could not achieve the target of having at least one centre for carrying out smear microscopy under the WHO global plan to stop TB 2011-2015.[4] In the backdrop of such sorry state of affairs, our laboratory was one of the very few in Pakistan with the capacity to carry out Drug Susceptibility Testing (DST) to first and second line anti-tuberculosis drugs. During the last decade or so MGIT 960 system has been extensively studied and validated for susceptibility testing of first line anti-tuberculosis drugs. The multicentre laboratory validation of the BACTEC MGIT 960 technique for testing susceptibilities of *M. tuberculosis* to classical second line drugs and newer anti-microbials has provided us with a guideline for resource poor countries like Pakistan to endeavor testing such compounds against our local isolates.[6]

It was found in this study that 3% of MDR-TB isolates turned out to be XDR-TB being resistant to injectable second line drugs AK and LEVO. Since the paucity of diagnostic facilities has already been documented by WHO, so the XDR cases found in this study could just be the tip of an iceberg in our country. Although total numbers of LEVO resistant MDR-TB cases were much higher (24%), hence the potential of further development of XDR-TB in our isolates is much more. Cases with XDR-TB may become virtually untreatable depending on the level of resistance to second line drugs and importantly the efficiency of health system in each given setting. Incorrect treatment of TB is the main risk factor for the development of resistance among TB cases and usually is associated with intermittent use of the drugs, errors in medical prescription, poor patient adherence and low quality of antituberculosis drugs.[8]

As 3% of our MDR-TB isolates were XDR-TB with MICs of AK 1.0 μg/ml and LEVO 2 μg/ml, it is imperative that we keep a close eye on the MICs of our MDR-TB isolates for these drugs with ever increasing threat of XDR-TB looming around us. In one of the previous study done in Pakistan, it was found that 2% of the MDR-TB isolated from central part of Pakistan (Lahore) were XDR-TB.[9] In Turkey, second line drug resistance rate of MDR-TB isolates to AK was 1.2% and 2.5% with LEVO.[10] Our isolates definitely have much higher rates of resistance to these two drugs. In India, the percentage of XDR-TB cases among MDR-TB was reported to be 3.7%.[11] The proportion of XDR-TB among MDR-TB cases was highest in Azerbaijan (Baku city, 12.8%), Belarus (11.9%), Latvia (16%), Lithuania (24.8%) and Tajikistan (Dushanbe city and Rudaki district (21%).[4] In one of the retrospective cohort study carried out recently, 72% of XDR-TB patients have been previously diagnosed as MDR-TB cases, highlighting the role of ineffective TB programs in generating XDR-TB.[12]

If we consider LEVO resistance rate in isolation, we found that 24% of MDR-TB isolates were resistant. Hence, the frequency of pre-XDR among MDR-TB isolates in this study was 24%. Pre-XDR rates reported from other countries include 16.7% in Nigeria, 12.1% in Poland, 31% in China and USA, 28% in Taiwan.[13-17] The high rate of this resistance observed in the study is not surprising in a setting where majority of the antibiotics are freely available everywhere in the country.[18] In addition, the antimicrobial resistance among other

bacteria is very high in our community as well with, for example 98% of *Neisseria gonorrhea* and 30% of *Salmonella enterica Serovar typhi* being resistant to FQs.[19,20]

The fact that treatment status record of patients with XDR and pre-XDR-TB isolates revealed that majority of patients had received FQs during the course of treatment is quite significant. Previous experiences with the use of FQs have shown that these compounds are susceptible for indiscriminate use for bacterial infections. The results of meta-analysis carried out recently show that patients who have been exposed to FQs prior to the diagnosis of TB are at higher risk of infection from FQs resistant TB than patients with TB who have no previous exposure to the drugs. Despite this documented risk, little effort is being done to restrict the use of FQs with only 7 of 15 European national guidelines issued for respiratory tract infections cautioning against misuse.[21]

In Pakistan, low rate of literacy and tendency of patients to frequently change their physicians and treatment facilities making it practically impossible to find out as to who has not received treatment in the past. There is every likelihood that patients who are bracketed in the category of no history of treatment may actually have been partially or inadequately treated and acquired drug resistance. As MDR and XDR-TB in Pakistan are on the rise, it is imperative to increase the awareness of local medical community as regards this emerging health problem. There are studies which have already highlighted the gap in knowledge and practice of physicians treating patients with TB.[22] The quantum of knowledge regarding drug sensitive TB has been found to be inadequate among the community practitioners who are the first to encounter the TB patients seeking treatment for their symptoms. In one study focusing General Practitioners (GPs), it was found that two-third of the prescriptions written for 60 kg man with newly diagnosed smear positive pulmonary TB were not in accordance with national guidelines and only 3% of the GPs knew all the five components of Directly Observed Treatment Short Course (DOTS).[22] In another study conducted in Pakistan, it was revealed that many doctors of various grades and seniority were not familiar with correct and proper description of MDR and XDR-TB. This study involved three tertiary care teaching hospitals in which medical specialists of different seniorities were surveyed and questioned regarding the basic knowledge of MDR and XDR-TB. The results revealed astonishingly lack of core knowledge about MDR and XDR-TB. Only around 40% correct responses were received for definition of MDR-TB while only 3% correct answers were recorded for XDR-TB.[23]

As 24 % of our MDR-TB isolates were resistant to LEVO and this surely is a worrying sign as cross-resistance among different members of FQs is of concern because they have a mode of action different from that of the classical first-line anti-TB drugs. Having been used widely for other infectious diseases, they are even available without prescription in several countries, increasing the burden of selective pressure and compromising their efficacy in the treatment of TB. The main target of FQs in *M. tuberculosis* is the DNA gyrase, encoded by gyrA and gyrB and mutations in two short regions known as “quinolone resistance-determining regions” have been associated with FQ resistance in *M. tuberculosis*.[24] Only very few centres reliably do testing for susceptibility to later-generation FQs, therefore, it is rarely checked and is known for very few of the isolates. In addition to inadequate treatment of TB, the challenging sociopolitical situation in Pakistan is likely to exacerbate this public health problem. The isolation of XDR-TB strains is not cause of concern in this area but also to be recognized as regional public health issue which requires national and international support.

Whereas we found 3% resistant isolates of AK and 24% of LEVO, nevertheless exact susceptibility percentages of these two antimicrobials has become extremely important due to WHO guidelines for the programmatic management of drug resistant TB.[25] According to this recommendation two of guidelines states "In the treatment of patients with MDR-TB, a FQ should be used”, whereas recommendation three states "in the treatment of patients with MDR-TB a later generation FQ should be used". It was due to this therapeutic reason that we used LEVO instead of earlier generation FQs in determining the *in vitro* susceptibility of MDR-TB.

It is high time that medical professionals dealing with cases of TB are cognizant of the status of MDR, pre-XDR and XDR in our own population. It is also extremely important that medical professionals should stress and strive to know the susceptibilities of *M. tuberculosis* to first and second line drugs before initiating the definitive therapy. Last but not the least is the issue of judicious use of antimicrobials specially FQs in other infections to ward off the escalating resistance in *M. tuberculosis*.

CONCLUSION

XDR-TB has started to emerge in MDR-TB isolates in our set up. The present study detected 3% of all MDR-TB isolates to be XDR. The worrying sign is the high frequency of pre-XDR-TB as we found 24% of isolates to be in this category. Urgent steps need to be taken to stem the tide of pre-XDR-TB in our population. It is thus recommended to develop facilities to carry out Drug Susceptibility Testing (DST) to monitor the status of pre-XDR and XDR in our population.

REFERENCES

1. Caminero JA, Torres A. Controversial topics in tuberculosis. *Eur Respir J* 2004; **24**:895-6.
2. Grzybowski S, Enarson DA. Results in pulmonary tuberculosis

patients under various treatment program conditions. *Bull Int Union Tuberc* 1978; **53**:70-5.

3. World Health Organization. Definitions and reporting framework for tuberculosis - 2013 revision. Geneva: *WHO*; 2013.
4. World Health Organization. Global tuberculosis report 2013. Geneva: *WHO*; 2013.
5. World Health Organization. Roadmap to prevent and combat drug-resistant tuberculosis. Geneva: *WHO*; 2011.
6. Rusch-Gerdes S, Pfyffer GE, Casal M, Chadwick M, Siddiqi S. Multicentre laboratory validation of the BACTEC MGIT 960 technique for testing susceptibilities of *Mycobacterium tuberculosis* to classical second line drugs and newer antimicrobials. *J Clin Microbiol* 2006; **44**:688-92.
7. Grace Lin SY, Desmond E, Siddiqi S. Multicentre evaluation of BACTEC MGIT 960 system for second line drug susceptibility testing of *Mycobacterium tuberculosis* complex. *J Clin Microbiol* 2009; **47**:3630-4.
8. Abubakar I, Zignol M, Falzon D. Drug-resistant tuberculosis: time for visionary political leadership. *Lancet Infect Dis* 2013; **13**:529-39.
9. Iqbal R, Shabbir I, Munir K, Chaudhry K, Qadeer E. The first and second line anti-TB drug resistance in Lahore. *Pak J Med Res* 2012; **51**:1-4.
10. Bekotre B, Hanzedaraglu T, Baylon O, Ozyurt M, Ozkutuk N, Satana D, *et al.* Investigation of extensive drug resistance isolates in multidrug resistance tuberculosis isolates. *Mikrobiyol Bul* 2013; **47**:59-70.
11. Porwal C, Kaushik A, Makkar N, Banawaliker JN, Hanif M, Singla R, *et al.* Incidence and risk factors for extensively drug resistant tuberculosis in Delhi region. *PloS One* 2013; **8**: e25529.
12. Dheda K, Shean K, Zumla A. Early treatment outcomes and HIV status of patients with extensively drug-resistant tuberculosis in South Africa: a retrospective cohort study. *Lancet* 2010; **375**:1798-807.
13. Daniel O, Osman E, Oladimeji O, Dairo OG. Pre-extensive drug resistant tuberculosis (Pre-XDR-TB) among MDR-TB patients in Nigeria. *Glo Adv Res J Microbiol* 2013; **2**:22- 8.
14. Kozinska M, Brzostek, Krawiecka D, Rybczynska M, ZofiaZwolska Z, Ugustynowicz-Kopec E . MDR, pre-XDR and XDR drug resistant tuberculosis in Poland in 2000-2009. *Pneumonol Alergol Pol* 2011; **79**:278-87.
15. Qi YC, Ma MJ, Li DJ, Chen MJ, Lu QB. Multidrug-resistant and extensively drug-resistant tuberculosis in multi-ethnic region, Xinjiang Uygur autonomous region, China. *PLoS ONE* 2012; **7**: e32103.
16. Banerjee R, Allen J, Westenhouse J, Oh P, Elms W, Desmond E. Extensively drug-resistant tuberculosis in California, 1993-2006. *Clin Infect Dis* 2008; **47**:450-7.
17. Wang JY, Lee LN, Lai HC, Wang SK, Jan IS, Yu CJ, *et al.* Fluoroquinolone resistance in *Mycobacterium tuberculosis* isolates:associated genetic mutations and relationship to antimicrobial exposure. *J Antimicrob Chemother* 2007; **59**: 860-65.
18. Butt ZA, Gilani AH, Nanan D, Sheikh AL, White F. Quality of pharmacies in Pakistan: a cross-sectional survey. *Int J Qual Health Care* 2007; **17**:307-13.
19. Jabeen K, Nizamuddin S, Irfan S, Khan E, Malik F, Zafar A. Increasing trend of resistance to penicillin, tetracycline, and fluoroquinolone resistance in *Neisseria gonorrhoeae* from Pakistan (1992-2009). *J Trop Med* 2011; **960**:501.
20. Hasan R, Zafar A, Abbas Z, Mehraj V, Malik F, Zaidi A. Antibiotic resistance among *Salmonella enterica, Serovars typhi* and *paratyphi A* in Pakistan (2001-2006). *J Infect Develp Countr* 2008; **2**:289-94.
21. Adriaenssens N, Coenen S, Versporten A. European Surveillance of Antimicrobial Consumption (ESAC): outpatient quinolone use in Europe (1997-2009). *J Antimicrob Chemother* 2011; **66**:47-56.
22. Khan JA, Malik A. Tuberculosis in Pakistan: are we losing the battle? *J Pak Med Assoc* 2003; **53**:320-1.
23. Ali W, Naseem A, Hani M, Shahzad M, Ali FM. Status of health professionals awareness about resistant tuberculosis. *Pak J Chest Med* 2010; **16**:4-8.
24. Sun Z, Zhang J, Zhang X, Wang S, Zhang Y, Li C. Comparison of gyrA gene mutations between laboratory-selected ofloxacin-resistant *Mycobacterium tuberculosis* strains and clinical isolates. *Int J Antimicrob Agents* 2008; **31**:115-21.
25. Falzan D, Jaramillo E, Shunemann HJ. WHO guidelines for the programmatic management of drug resistant tuberculosis: 2011 update. *Eur Resp J* 2011; **38**:516-28.