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Analysis of Multi Drug Resistant *Mycobacterium tuberculosis* Clinical Isolates for Susceptibility to Linezolid and Verapamil using MGIT 960

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Original Research Article

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Abstract: Pakistan stands among the top five highest tuberculosis burden countries. Emergence of multi drug resistant *Mycobacterium tuberculosis* limited the spectrum of drugs of choice to treat tuberculosis. Among several, the activation of efflux pumps is one of mechanisms that mediate drug resistance in *M. tuberculosis*. Here, we tested efflux pump inhibitor verapamil and third line tuberculosis drug linezolid for antimicrobial activity against locally isolated multi drug resistant *M. tuberculosis* isolates using MGIT 960. *M. tuberculosis* isolates were collected from two tertiary care hospitals based in Lahore. Out of 100 isolates, 56 % (n=56) were found to be multi drug resistant (MDR). Out of MDR-TB, 5.36% (n=3) were additionally resistant to linezolid and 41.07% (n=17) were resistant to amikacin. One moxifloxacin resistant isolate was identified. In linezolid resistant strains, *rplC* gene was sequenced where *rplC* T640C mutation was identified in two of three linezolid resistant strains. The minimum inhibitory concentration of verapamil in 84.5% isolates was 256 µg/ml and the growth of remaining 16.5% isolates was inhibited by verapamil at the concentration of 512 µg/ml. Assessment of synergism between verapamil and other drugs including rifampicin, isoniazid, linezolid, amikacin and moxifloxacin revealed that verapamil at the concentration of 256 µg/ml inhibited the growth of MDR-TB isolates in presence of respective drugs. Our findings suggest that linezolid resistance have started emerging in local isolates. Further studies are required at higher level to identify the exact mechanism.

Keywords: Tuberculosis, Multi drug resistance, Linezolid, Verapamil, Efflux pumps.

INTRODUCTION

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is one of the major public health issues in Pakistan. Pakistan stands among the five top TB burden countries in the world . Every year approximately, 0.42 million new cases are reported in the country [2].

TB infection is considered as one of the most difficult to treat infections. Emergence of Multidrug-resistance in tuberculosis (MDR-TB) has further intensified the situation globally. Such a resistance can occur to the most of drugs prescribed to treat TB patients including rifampicin, isoniazid, pyrazinamide (PZA) and ethambutol [1] which are used as combinational therapy to treat *M. tuberculosis* infections routinely. Hence, resistance to any of these drugs makes infection difficult to treat. Also, the treatment becomes delayed with toxic effects, resulting in poor control of disease [9]. In 2010, MDR TB burden was 0.650 million globally which reflects a major threat to TB control at global level [5]. Pakistan stands among four top countries with respect to prevalence of multidrug-resistant TB (MDR-TB) [9].

There are several mechanisms acquired by *M. tuberculosis* to acquire resistance against drugs such as mutation in target genes, horizontal transfer of drug resistance genes, altered cell wall permeability to drugs and the activation of drug efflux pumps [2, 1]. Resistance due to impermeability of cell wall for the drug or pumping out the drug through efflux pumps is classified under intrinsic resistance [1-21].

Drug transporters have become an important part of drug resistance panorama, which use their ability to decrease the concentration of drug inside the cell and therefore increase the low specificity requirements. Hence such transporters could easily become the first line of defense against TB drug.

Efflux pumps are outer or inner membrane spanning proteins that export products from bacterial and eukaryotic cells. Multiple products and substrates including antibiotics are exported out from the cell to the outer environment. Although several types of drug efflux systems operate in bacteria but molecular details

of operating mechanisms for individual drugs are not well characterized so far [1, 20, 2]. Initially, operating function of efflux pumps where multiple drugs are extruded by a transporter was characterized in eukaryotic cells [18]. Later on, it was unraveled that similar multidrug efflux pumps are operational in several bacteria [2]. In case of *Mycobacterium*, several mechanisms of efflux pump activation have been reported [2, 13, 3]. *Mycobacterium smegmatis* has been extensively used as the model organism to understand mechanisms of extruding drug out through drug efflux pumps [13, 6, 12, 11].

Certain compounds have been known with potential to inhibit efflux pumps. Such drug pumps inhibitor can be a candidate compounds to test activity as antimicrobial agents. Particularly, the synergetic use of drug pump inhibitors along with antibiotic therapy might be more effective option to treat tuberculosis.

Certain compounds are already in use in clinical practice for other purposes, such as verapamil, reserpine and omeprazole are capable of inhibiting efflux mechanisms in several eukaryotic and prokaryotic cells [1]. Use of reserpine, an efflux pump inhibitor, decreased isoniazid resistance in *M. tuberculosis*, suggesting a role of efflux pump in isoniazid transport [4].

Verapamil is an effective efflux pump inhibitor that appears promising as adjunctive chemotherapy for TB. There have been few studies in experimental mice suggesting that the use of verapamil along with rifampicin enhanced rifampicin levels inside bacteria. *M. tuberculosis* develops bacterial efflux pump mediated tolerance to isoniazid and rifampacin following macrophage residence [1]. This macrophage induced tolerance was inhibited by verapamil, which has shown to accelerate bacterial killing in mice models, infected with drug resistant or drug sensitive TB [12, 1]. Moreover, inhibition of efflux pumps of *M. tuberculosis* by verapamil has been shown to reduce the macrophage-induced bacterial drug tolerance in lung granulomas. However the synergetic effect of verapamil with most of antibiotics used to treat tuberculosis remains to elucidate. Here, we estimated current status of multi drug resistance in two tertiary care hospitals as well as in outdoor patients based in Lahore, Pakistan. We further tested MDR isolates for the susceptibility to third line anti tuberculosis drug linezolid and efflux pump inhibitor verapamil.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Well-isolated confirmed *M. tuberculosis* isolates were collected from Gulab Devi Chest Hospital Lahore, (n=41), Mayo Hospital Lahore (n=31) and selected outdoor private laboratories in Lahore (n= 28). All the strains included in the study are listed in table S1. All strains were reconfirmed by standard protocol of Ziehl–Neelsen (ZN) staining and subsequently sub cultured in MGIT 960 as described previously.

Drug susceptibility testing (DST)

All culture positive MGIT 960 vials were incubated manually at 37^{o}C for four days by following MGIT 960 manual protocol. On fourth day, drug susceptible testing was performed using following method. BACTEC 960 SIRE Supplement (0.8 ml) was added to each MGIT tube. Aseptically, 0.1 ml of properly reconstituted drug was added into each tube in following concentrations: rifampicin 1.0 ug/ml, isoniazid 0.1 ug/ml, amikacin, 1.0 µg/ml; moxifloxacin 0.125µg/ml [3] and linezolid, 1.0 µg/ml [3]. Aseptically 0.5 ml of the well-mixed culture suspensions diluted with water (1:100) were added into each of the drug containing tubes using a pipette except growth control (GC) tube. For control, test culture suspensions were diluted to 50 times with normal saline. Inoculated MGIT vials were mixed well by gently inverting the tubes several times. Inoculated MGIT vials were loaded in MGIT 960 according to MGIT 960 manual protocol. The instrument interpreted the susceptibility results at the time when the growth unit (GU) in growth control reached to 400 units (within 4-13 days). At this point, the GU values of the drugs vial were evaluated according to following criteria.

S = Susceptible – the GU of the drug tube is less than 100
R = Resistant – the GU of the drug tube is 100 or more.

MDR cases were refreshed for second line and third line drug susceptibility testing.

Synergetic effect of Verapamil along with antibiotics

In order to determine synergism of verapamil along with other antibiotics, thirty three MDR isolates were selected. DST at different concentrations of verapamil (64, 128, 256 and 512 ug/ml) in the presence and absence of antibiotics at following concentration was performed: rifampicin 1.0 ug/ml, isoniazid 1.0 ug/ml, amikacin, 1.0 µg/ml, moxifloxacin 0.125µg/ml and linezolid 1.0 µg/ml.

Molecular Biology Techniques

DNA was isolated from three linezolid resistant strains and one linezolid sensitive control. The *rplC* gene was amplified by conventional PCR using primers *rplC*_F (GCTGCGGCTGGACGACTC and *rplC*_R (CTCTTGCGCAGCCATCACTTC). The conditions used were (i) initial denaturation at 95°C for 15 min, (ii) denaturation at 94°C for 30 s, (iii) annealing at 65°C for 30 s, (iv) elongation for 30 s at 72°C, and (v) terminal elongation for 10 min at 72°C. There were thirty five cycles from step ii to iv. The DNA of PCR products was sequenced by Sanger Sequencing. Multi sequence alignment was performed using NCBI Omega Blast Software.

RESULTS

Frequency of dug resistance in *M. tuberculosis*

In total, one hundered well isolated consecutive *Mycobacterium tuberculosis* isolates collected from two TB centers between February 2016 and February 2017 and randomly collected samples from private laboratories during same duration were included in the study. Among hundred isolates, forty nine isolates were from Gulab Devi Hospital Lahore, thirty one isolates were from Mayo hospital Lahore and twenty eight isolates were from selected private laboratories based in Lahore (Table S1).

Screening of *M. tuberculosis* isolates for drug resistance identified 55 multi drug resistant (MDR) and 1 extensive drug resistant (XDR) isolates. Isolates resistant to 1st line drugs isoniazid and rifampicin were declared as MDR and MDR isolates additionally resistant to any fluoroquinolone and at least one of the injectable drugs (amikacin, capreomycin or kanamycin) were declared as XDR . Out of these 56 MDR isolates, 5.4% (n=3) were resistant to linezolid, 41% (n=23) were resistant to amikacin and 1.8% (n=1) were resistant to moxifloxacin. Among non MDR isolates, 15.5% (n=7) were resistant to amikacin (Table-1, Fig 1A, 1B). It is believed that resistance to rifampicin and isoniazid in *M. tuberculosis* co exist. However, the isolate 3174G was found to be resistant to rifampicin but sensitive to isoniazid.

Centre wise distribution of MDR TB isolates

The frequency MDR-TB in Gulab Devi Hospital is found to be alarmingly high where thirty four out of forty one (83%) isolates found to be MDR isolates whereas, the frequency of MDR isolates in Mayo Hospital is found to be 45%. In contrast, isolates collected from outside two major TB centers comprise of only 14% MDR cases (Table 1, Fig 1C). Our findings suggest that the frequency es of multi drug resistant isolates vary significantly among TB centers and outdoor patients.

Point mutations in *rplC* encoding gene among linezolid resistant isolates

The DNA fragment encoding *rplC* was amplified from three linezolid resistant and one linezolid sensitive isolates by conventional PCR. The PCR product was sequenced to identify mutations associated with linezolid resistance in linezolid resistant isolates. Multi sequence alignment of *rplC* encoding gene revealed that strain C71 and strain C66 carry a point mutation at thiamine 460 where thiamine is substituted by cytosine. The T460C mutation in *rplC* has been reported to mediate linezolid resistance in *M. tuberculosis*. Interestingly, *M. tuberculosis* C320 isolate although phenotypically linezolid resistant but contains *rplC* gene identical to *rplC* of linezolid sensitive control (Fig-3). This finding suggests that molecular mechanism leading to linezolid resistance other than mutation in *rplC* encoding gene exists in C320 isolate.

Minimum inhibitory concentration of verapamil

Verapamil in different concentrations was tested to assess the effect on the growth of multi drug resistant isolates. Verapamil at the concentration of 64 µg/ml and 128 µg/ml did not inhibit the growth of all thirty three tested isolates. However, Verapamil at the concentration of 256 µg/ml inhibited the growth of twenty nine out of thirty three isolates. In short, minimum inhibitory concentration of verapamil in 84.5% of tested isolates is found to be 256µg/ml. The growth of remaining 15.5% isolates (n=4) was inhibited by Verapamil at the concentration of 512µg/ml (Table S2, S3, S4,S5 and S6).

Effect of verapamil on rifampicin resistance in multi-drug resistant *M. tuberculosis* isolates

An isolate was declared as rifampicin resistant if it grew upon the treatment of 1µg/ml of rifampicin using MGIT 960. The effect of verapamil to overcome rifampicin resistance was tested in thirty three rifampicin resistant isolates. For that, rifampicin resistant isolates were treated with various concentrations of verapamil in the presence of 1µg/ml rifampicin. Verapamil at the concentration of 256 µg/ml inhibited the growth of all thirty three tested isolates in the presence of 1ug/ml rifampicin. However, verapamil alone at this concentration inhibited the growth of 84.5% isolates (Fig-3A, Table S2). These finding indicates that verapamil exhibits a synergetic effect along with rifampicin to overcome rifampicin resistance in 15.5% population of tested isolates.

Effect of verapamil on isoniazid resistance in isoniazid resistant *M. tuberculosis* isolates

An isolate was declared as resistant to isoniazid if it grew upon the treatment of isoniazid at the concentration of 0.1ug/ml. The effect of verapamil to overcome isoniazid resistance was tested in thirty three isolates. For that, isoniazid resistant isolates were treated with various concentrations of verapamil in the presence of 0.1ug/ml isoniazid. Verapamil at the concentration of 256 ug/ml inhibited the growth in all thirty three tested isolates in the presence of 0.1ug/ml isoniazid. However, verapamil alone at this concentration inhibited the growth of 84.5% isolates (Fig-3B, Table-S3). These findings suggest that verapamil exhibits a synergetic effect along with isoniazid to overcome rifampicin resistance in 15.5% population of tested isolates.

Effect of verapamil on amikacin resistance in amikacin resistant *M. tuberculosis* isolates

Thirteen out of thirty three isolates were found to be amikacin resistant. An isolate is declared as resistant to amikacin if it grew upon the treatment of 1µg/ml amikacin. The effect of verapamil to overcome amikacin resistance was tested in thirteen amikacin

resistant isolates. For that, amikacin resistant isolates were treated with various concentrations of verapamil in the presence of 1µg/ml amikacin. However, the synergetic effect of verapamil and amikacin to overcome amikacin resistance was not observed in any of tested isolates (Fig-3C, Table-S4).

Effect of verapamil on moxifloxacin resistance in moxifloxacin resistant *M. tuberculosis* isolate

One out of thirty three isolates was found to be moxifloxacin resistant. An isolate was declared as moxifloxacin resistant if it grew upon the treatment of moxifloxacin at the concentration of 2µg/ml. The effect of verapamil to overcome moxifloxacin resistance was tested in moxifloxacin resistant isolate. For that isolate was treated with various concentrations of verapamil in the presence of 2 µg/ml moxifloxacin. Minimum inhibitory concentration of verapamil in moxifloxacin resistant isolate was 256µg/ml. Also, in the presence of 2µg/ml moxifloxacin, verapamil at the concentration of 256 µg/ml inhibited the growth of moxifloxacin resistant isolate. Verapamil at lower concentrations did not affect the moxifloxacin resistance (Fig-3D, Table-S5). In short, synergetic effect of verapamil and moxyfloxacin in tested experimental conditions was not observed.

Effect of verapamil on linezolid reistance in linezolid resistant *M. tuberculosis* isolates

Three out of thirty three isolates were found to be linezolid resistant. An isolate was declared as linezolid resistant if it grew upon the treatment of linezolid at the concentration of 1µg/ml. The effect of verapamil to overcome linezolid resistance was tested in three linezolid resistant isolates. For that isolates were treated with various concentrations of verapamil in the presence of 1µg/ml linezolid. Minimum inhibitory concentration of verapamil in linezolid resistant isolates was 256 µg/ml. Also, in the presence of 1 ug/ml of linezolid, verapamil at the concentration of 256 µg/ml inhibited the growth of all three linezolid resistant isolates. Verapamil at lower concentrations did not affect the linezolid resistance (Fig 3E, Table S6). In short, synergetic effect of verapamil and linezolid in tested experimental conditions was not observed.

DISCUSSION

A systematic country wide surveillance of MDR and XDR *M. tuberculosis* is lacking in Pakistan. However, the introduction of modern *M. tuberculosis* drug susceptibility testing techniques in recent years makes it convenient to determine drug resistance pattern. For example, Batec MGIT 960 has now been frequently used to determine susceptibility testing of first line and second line anti tuberculosis drugs [11]. We used Bactec MGIT 960 for the susceptibility testing of first, second and third line anti tuberculosis drugs at critical concentrations. In general, we found relatively higher burden of MDR-TB (54%) in Lahore. However, XDR-TB burden is relatively lower as compared to previously reported in Karachi. Gulab Devi Hospital shares a major part of MDR TB burden where MDR-TB frequency (83%) is found to be alarmingly high. There is a need to further investigate the causes of such a high MDR TB burden in Gulab Devi hospital. It might be because of the fact that complicated and difficult to treat cases are referred to Gulab Devi Hospital as it is one of the prime and oldest TB centers in Lahore.

We found 23% (n=23) amikacin resistant isolates which is remarkably high frequency of amikacin resistance as compared to 3% previously reported in Pakistan [4]. Previous data on Amikcain resistant *M. tuberculosis* in Pakistan is rare and therefore, these findings highlight the emergence of amikacin resistance in the country. However, in order to get more accurate status of amikacin resistance in the country, further analysis on larger sample size is required.

Although linezolid is a third line anti TB drug and is not in routine use but we found cases of 3% linezolid resistant *M. tuberculosis.* Previously, 6/102 (5.9%) linezolid resistant was reported in Karachi [6] and 4.0% in Rawalpindi. Altogether, these data reveal a uniform pattern of linezolid resistance in country. In other countries of the region, prevalence of linezolid resistance is relatively higher. For example, two independent studies in China reported 26.7% [4] and 10.8% linezolid resistant cases among MDR-TB isolates [3]. Probably, high frequency of linezolid resistant *M. tuberculosis* in China is a consequence of the frequent use of linezolid to treat tuberculosis. T460C mutation in *rplC* gene is associated with linezolid resistance [5]. However, we found such a mutation in two of three linezolid resistance strains. This finding suggests that remaining strain harbors linezolid resistance through some alternative mechanisms.

It has been proposed that verapamil enhances the rifampicin levels inside *M. tuberculosis* and develops bacterial efflux pump mediated tolerance to isoniazid and rifampacin, following macrophage residence [1]. This macrophage induced tolerance was inhibited by verapamil, which has shown to accelerate bacterial killing in mice model, infected with drug resistant or drug sensitive TB and have decreased relapse rate of disease and shortening the duration of the treatment [12, 1]. However, our findings indicate that Verapamil at the concentrations of 128 µg/ml or below is ineffective to overcome resistance to Rifampicin, Isonaiazid, Amikacin, Moxifloxacin and Linezolid. All of thirty-three MDR-MTB isolates became sensitive to both Rifampicin and Isoniazid in combination with Verapamil only at the concentarion of 256ug/ml and above. Physiologically, such a high

concentration of Verapamil is relevant to the therapeutic use.

Table-1: Drug resistance pattern in *M. tuberculosis* isolates

S. No	Isolate ID	Resistant to	MDR/XDR/Non-MDR
1	1411M	Rifampicin, Isoniazid	MDR
2	1482M	Amikacin	Non-MDR
3	1480M	-	Non-MDR
4	635M	-	Non-MDR
5	618M	-	Non-MDR
6	1488M	-	Non-MDR
7	1484M	Rifampicin , Isoniazid	MDR
8	1195M	-	Non-MDR
9	1292M	Amikacin	Non-MDR
10	1292M	-	Non-MDR
11	1234M	Rifampicin, Isoniazid , Amikacin	MDR
12	1295M	-	Non-MDR
13	2747G	Rifampicin, Isoniazid, Moxifloxacin, Amikacin,	XDR
14	3210G	Rifampicin, Isoniazid , Amikacin	MDR
15	3144G	Amakicin	Non-MDR
16	3131G	Rifampicin, Isoniazid, Amakicin	MDR
17	3174G	Isoniazid	Non-MDR
18	3243G	Rifampicin, Isoniazid	MDR
19	2801G	Rifampicin, Isoniazid, Amikacin	MDR
20	2867G	Rifampicin, Isoniazid , Amikacin	MDR
21	3212G	Rifampicin, Isoniazid , Amikacin	MDR
22	3169G	Rifampicin, Isoniazid	MDR
23	1274M	-	Non-MDR
24	1833M	-	Non-MDR
25	1857M	-	Non-MDR
26	1801M	-	Non-MDR
27	1852M	Rifampicin, Isoniazid	MDR
28	635M	-	Non-MDR
29	1780M	-	Non-MDR
30	1815M	Rifampicine, Isoniazid, Amikacin	MDR
31	1293M	-	Non-MDR
32	1405M	-	Non-MDR
33	2729G	-	Non-MDR
34	2915G	Rifampicin, Isoniazid	MDR
35	2671G	Rifampicin, Isoniazid	MDR
36	2457G	Rifampicin, Isoniazid	MDR
37	2682G	Rifampicine, Isoniazid, Amikacin	MDR
38	2556G	Rifampicin, Isoniazid	MDR
39	2680G	Rifampicin, Isoniazid	MDR
40	2952G	Rifampicin, Isoniazid	MDR
41	2458G	Rifampicin, Isoniazid	MDR
42	2678G	-	Non-MDR
43	1993G	Rifampicin, Isoniazid	MDR
44	2013G	Rifampicine, Isoniazid, Amikacin	MDR
45	1939G	Rifampicin, Isoniazid	MDR
46	2458G	Rifampicin, Isoniazid	MDR
47	2918G	Rifampicin, Isoniazid	MDR
48	2694G	Rifampicin, Isoniazid	MDR
49	2800G	Rifampicine, Isoniazid, Amikacin	MDR
50	2456G	Rifampicin, Isoniazid	MDR
51	2684G	Rifampicin, Isoniazid	MDR
52	2916G	Rifampicin, Isoniazid	MDR
53	2136G	Rifampicin, Isoniazid	MDR

54	2143G	Rifampicine, Isoniazid, Amikacin	MDR
55	1909G	-	Non-MDR
56	1968G	Rifampicin, Isoniazid	MDR
57	2524G	Rifampicin, Isoniazid	MDR
58	2011G	Rifampicin, Isoniazid	MDR
59	2682G	Rifampicine, Isoniazid, Amikacin	MDR
60	2620G	Rifampicin, Isoniazid	MDR
61	2363G	Rifampicine, Isoniazid, Amikacin	MDR
62	1971G	Rifampicin, Isoniazid	MDR
63	82A	-	Non-MDR
64	1933M	Rifampicine, Isoniazid, Amikacin	MDR
65	320G	Rifampicin, Isoniazid, Amikacin, Linezolid	XDR
66	1906M	Rifampicin, Isoniazid	MDR
67	2141M	Rifampicin, Isoniazid	MDR
68	71M	Rifampicin, Isoniazid, Linezolid	MDR
69	1908M	Rifampicin, Isoniazid	MDR
70	19009M	Rifampicin, Isoniazid	MDR
71	19008M	Rifampicin, Isoniazid	MDR
72	19033M	Rifampicin, Isoniazid	MDR
73	19058M	Rifampicin, Isoniazid	MDR
74	66A	Rifampicin, Isoniazid, Linezolid	MDR
75	79A	-	Non-MDR
76	89A	-	Non-MDR
77	93A	-	Non-MDR
78	84A	-	Non-MDR
79	95A	-	Non-MDR
80	94A	-	Non-MDR
81	83A	-	Non-MDR
82	90A	-	Non-MDR
83	88A	-	Non-MDR
84	87A	Amakicin	Non-MDR
85	102A	Amakicin	Non-MDR
86	80A	-	Non-MDR
87	831A	-	Non-MDR
88	86A	Amakicin	Non-MDR
89	79A	-	Non-MDR
90	90A	-	Non-MDR
91	81A	-	Non-MDR
92	87A	-	Non-MDR
93	84A	Rifampicin, Isoniazid	MDR
94	44A	Rifampicin, Isoniazid	MDR
95	55A	-	Non-MDR
96	76A	Amikacin	Non-MDR
97	73A	-	Non-MDR
98	170A	Rifampicin, Isoniazid	MDR
99	144A	-	Non-MDR
100	148A	-	Non-MDR

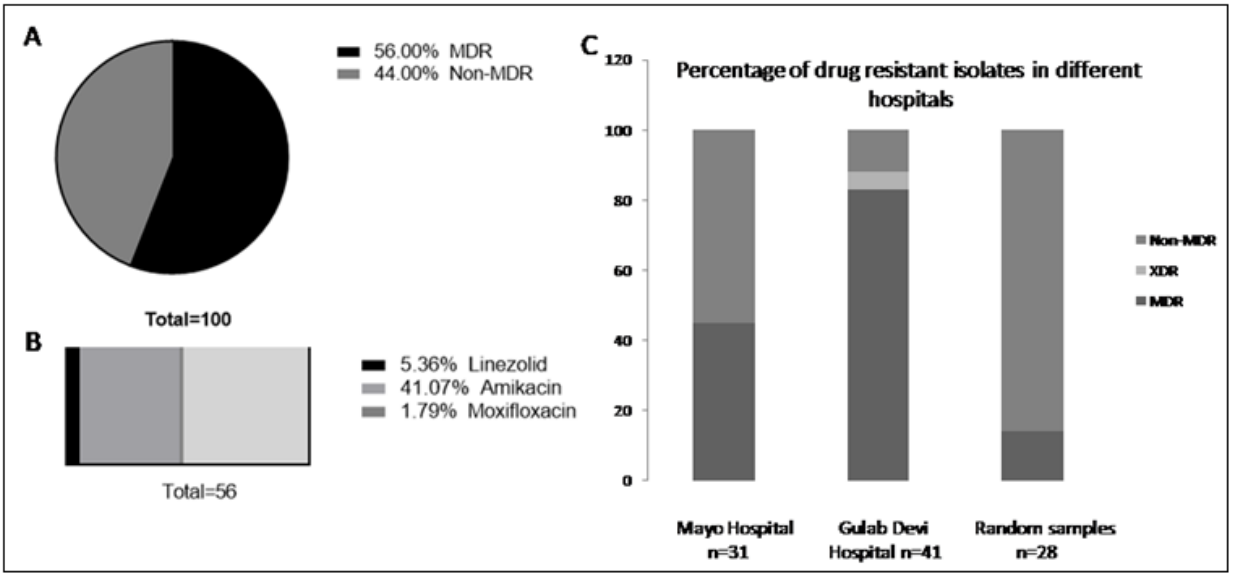

Fig-1: Pattern of multi drug resistance in *M. tuberculosis* isolates

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CLUSTAL O(1.2.4) multiple sequence alignment

Control     TGGGCAAGAGTTGACCGCGGAGATCTTCGCCGATGGCAGCTACGTCGATGTGACGGGTAC
C71         TGGGCAAGAGTTGACCGCGGAGATCTTCGCCGATGGCAGCTACGTCGATGTGACGGGTAC
C66         TGGGCAAGAGTTGACCGCGGAGATCTTCGCCGATGGCAGCTACGTCGATGTGACGGGTAC
C320        TGGGCAAGAGTTGACCGCGGAGATCTTCGCCGATGGCAGCTACGTCGATGTGACGGGTAC
            ************************************************************

Control     CTCCAAGGGCAAAGGTTTCGCCGGCACCATGAAGCGGCACGGCTTCCGCGGTCAGGGCGC
C71         CTCCAAGGGCAAAGGTTTCGCCGGCACCATGAAGCGGCACGGCTTCCGCGGTCAGGGCGC
C66         CTCCAAGGGCAAAGGTTTCGCCGGCACCATGAAGCGGCACGGCTTCCGCGGTCAGGGCGC
C320        CTCCAAGGGCAAAGGTTTCGCCGGCACCATGAAGCGGCACGGCTTCCGCGGTCAGGGCGC
            ************************************************************

Control     CAGTCACGGTGCCCAGGCGGTGCACCGCCGTCCGGGCTCCATCGGCGGATGTGCCACGCC
C71         CAGTCACGGTGCCCAGGCGGTGCACCGCCGTCCGGGCTCCATCGGCGGACGTGCCACGCC
C66         CAGTCACGGTGCCCAGGCGGTGCACCGCCGTCCGGGCTCCATCGGCGGACGTGCCACGCC
C320        CAGTCACGGTGCCCAGGCGGTGCACCGCCGTCCGGGCTCCATCGGCGGATGTGCCACGCC
            ********************************************** **********

Control     GGCGCGGGTGTTCAAGGGCACCCGGATGGCCGGGCGGATGGGCAATGACCGGGTGACCGT
C71         GGCGCGGGTGTTCAAGGGCACCCGGATGGCCGGGCGGATGGGCAATGACCGGGTGACCGT
C66         GGCGCGGGTGTTCAAGGGCACCCGGATGGCCGGGCGGATGGGCAATGACCGGGTGACCGT
C320        GGCGCGGGTGTTCAAGGGCACCCGGATGGCCGGGCGGATGGGCAATGACCGGGTGACCGT
            ************************************************************

Control     TCTTAACCTTTTGGTGCATAAGGTCGATGCCGAGAACGGCGTGCTGCTGATCAAGGGTGC
C71         TCTTAACCTTTTGGTGCATAAGGTCGATGCCGAGAACGGCGTGCTGCTGATCAAGGGTGC
C66         TCTTAACCTTTTGGTGCATAAGGTCGATGCCGAGAACGGCGTGCTGCTGATCAAGGGTGC
C320        TCTTAACCTTTTGGTGCATAAGGTCGATGCCGAGAACGGCGTGCTGCTGATCAAGGGTGC
            ************************************************************

Control     GGTTCCTGGCCGCACCGGTGGACTGGTCATGGTCCGCAGTGCGATCAAACGAGGTGAGAA
C71         GGTTCCTGGCCGCACCGGTGGACTGGTCATGGTCCGCAGTGCGATCAAACGAGGTGAGAA
C66         GGTTCCTGGCCGCACCGGTGGACTGGTCATGGTCCGCAGTGCGATCAAACGAGGTGAGAA
C320        GGTTCCTGGCCGCACCGGTGGACTGGTCATGGTCCGCAGTGCGATCAAACGAGGTGAGAA
            ************************************************************

Control     GTGA
C71         GTGA
C66         GTGA
C320        GTGA
            ****
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Fig-2: Multi sequence alignment of *rplC* in linezolid resistant isolates

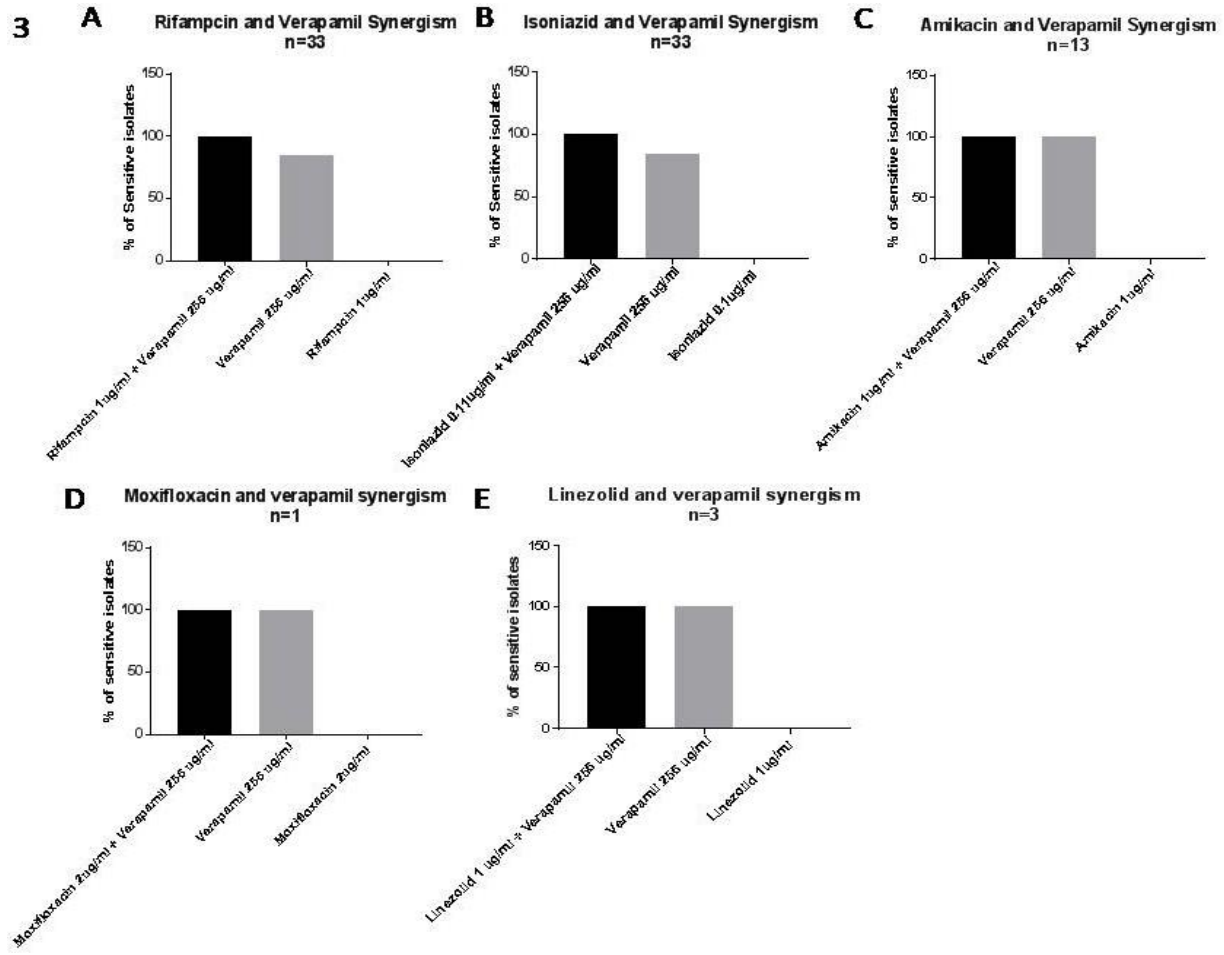

Fig-3: Bar diagram showing the number of *M. tuberculosis* isolates sensitive to verapamil.

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Disclosure Statement

No competing financial interests exist.

REFERENCES

1. Adams, K. N., Takaki, K., Connolly, L. E., Wiedenhoft, H., Winglee, K., Humbert, O., ... & Ramakrishnan, L. (2011). Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. *Cell*, *145*(1), 39-53.
2. Afridi, S. P., Siddiqui, R. A., Rajput, A., & Alam, S. N. (2016). Spectrum of abdominal-tuberculosis in emergency surgery: 100 cases at a tertiary care Centre Dow University of Health Sciences and Civil Hospital Karachi, Pakistan. *Stoma*, *34*, 34.
3. Ahmed, I., Jabeen, K., Inayat, R., & Hasan, R. (2013). Susceptibility testing of extensively drug resistant and pre-extensively drug resistant Mycobacterium tuberculosis against levofloxacin, linezolid, and amoxicillin/clavulanate. *Antimicrobial agents and chemotherapy*, AAC-02020.
4. Ali, S. M., Powers, R., Beorse, J., Naureen, F., Noor, A., Anjum, N., ... & Anderson, R. (2016). ODK Scan: Digitizing Data Collection and Impacting Data Management Processes in the Tuberculosis Control Program of Pakistan.
5. Altare, F., Durandy, A., Lammas, D., Emile, J. F., Lamhamedi, S., Le Deist, F., ... & Jeppsson, O. (1998). Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. *Science*, *280*(5368), 1432-1435.
6. Ambudkar, S. V., Dey, S., Hrycyna, C. A., Ramachandra, M., Pastan, I., & Gottesman, M. M. (1999). Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annual review of pharmacology and toxicology*, *39*(1), 361-398.
7. Ambudkar, S. V., Lelong, I. H., Zhang, J., Cardarelli, C. O., Gottesman, M. M., & Pastan, I. (1992). Partial purification and reconstitution of the

human multidrug-resistance pump: characterization of the drug-stimulatable ATP hydrolysis. *Proceedings of the National Academy of Sciences*, *89*(18), 8472-8476.

8. Beckert, P., Hillemann, D., Kohl, T. A., Kalinowski, J., Richter, E., Niemann, S., & Feuerriegel, S. (2012). rplC T460C identified as a dominant mutation in linezolid resistant Mycobacterium tuberculosis strains. *Antimicrobial agents and chemotherapy*, AAC-06227.
9. Colangeli, R., Helb, D., Sridharan, S., Sun, J., Varma-Basil, M., Hazbón, M. H., ... & Sacchettini, J. C. (2005). The Mycobacterium tuberculosis iniA gene is essential for activity of an efflux pump that confers drug tolerance to both isoniazid and ethambutol. *Molecular microbiology*, *55*(6), 1829-1840.
10. Corbett, E. L., Watt, C. J., Walker, N., Maher, D., Williams, B. G., Raviglione, M. C., & Dye, C. (2003). The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. *Archives of internal medicine*, *163*(9), 1009-1021.
11. de Knegt, G. J., Bruning, O., Marian, T., de Jong, M., van Belkum, A., Endtz, H. P., ... & de Steenwinkel, J. E. (2013). Rifampicin-induced transcriptome response in rifampicin-resistant Mycobacterium tuberculosis. *Tuberculosis*, *93*(1), 96-101.
12. Rossi, E. D., Aínsa, J. A., & Riccardi, G. (2006). Role of mycobacterial efflux transporters in drug resistance: an unresolved question. *FEMS microbiology reviews*, *30*(1), 36-52.
13. Ghafoor, T., Ikram, A., Abbasi, S. A., Zaman, G., Ayyub, M., Palomino, J. C., ... & Martin, A. (2015). Sensitivity Pattern of Second Line Anti-Tuberculosis Drugs against Clinical Isolates of Multidrug Resistant Mycobacterium Tuberculosis. *Journal of the College of Physicians and Surgeons Pakistan*, *25*(4), 250-253.
14. Ghoshal, T. K., Mohini, M., & Singh, G. P. (1998). Effect of Bentonite supplementation on Nitrogen metabolism from diets containing urea in cattle.
15. Gupta, H., PawaT, D., Riva, A., & Bombardelli, E. (2010). Thirty-Ninth Annual.
16. Hasan, Z., Ali, A., Mcnerney, R., Mallard, K., Hill-Cawthorne, G., Coll, F., Nair, M., Pain, A., Clark, T. G., & Hasan, R. (2015). Whole genome sequencing-based characterization of extensively drug resistant (xdr) strains of mycobacterium tuberculosis from pakistan. *International Journal of Mycobacteriology*, 4:11-12.
17. Heysell, S. K., Pholwat, S., Mpagama, S. G., Pazia, S. J., Kumburu, H., Ndusilo, N., Gratz, J., Houpt, E. R., & Kibiki, G. S. (2015). Sensititre mycotb plate compared to bactec mgit 960 for first-and second-line antituberculosis drug susceptibility testing in tanzania: A call to operationalize mics. *Antimicrobial agents and chemotherapy*, 59 (11):7104-7108.
18. Hopewell, P. C. (2016). Treatment of tuberculosis. *Tuberculosis: The Essentials*, 237:113.
19. Jarlier, V., & Nikaido, H. (1994). Mycobacterial cell wall: structure and role in natural resistance to antibiotics. *FEMS microbiology letters*, *123*(1-2), 11-18.
20. Javaid, A., Khan, M. A., Khan, M. A., Mehreen, S., Basit, A., Khan, R. A., Ihtesham, M., Ullah, I., Khan, A., & Ullah, U. (2016). Screening outcomes of household contacts of multidrug-resistant tuberculosis patients in peshawar, pakistan. *Asian Pacific journal of tropical medicine*, 9 (9):909-912.
21. Krüüner, A., Yates, M. D., & Drobniewski, F. A. (2006). Evaluation of MGIT 960-based antimicrobial testing and determination of critical concentrations of first-and second-line antimicrobial drugs with drug-resistant clinical strains of Mycobacterium tuberculosis. *Journal of clinical microbiology*, *44*(3), 811-818.