MOLECULAR DETECTION OF MULTI DRUG RESISTANT TUBERCULOSIS (MDR-TB) IN MDR-TB PATIENTS' ATTENDANT IN NORTH WESTERN PAKISTAN

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ABSTRACT

Objective: To determine the drugs susceptibility pattern of mycobacterium tuberculosis (M.TB) in multi-drug resistant tuberculosis (MDR-TB) patients' attendants in North Western, Pakistan.
Study Design: Cross sectional study.
Place and Duration of Study: This study was conducted at Peshawar Tuberculosis Research Laboratory (PTRL), Provincial TB Control Program Hayatabad Medical Complex Peshawar, (KP) from August 2013 to March 2014.
Material and Methods: A cross sectional study in which four hundred and eighty sputum samples from MDR-TB patients' attendants were processed for the detection of M.TB through Ziehl-Neelsen staining, Lowenstein-Jensen, BACTEC MGIT-960 culture and line probe assay.
Results: Out of 480 samples, 06 (2.1%) were found positive for M.TB through Ziehl-Neelsen staining while 10 (2.8%) were positive through LJ and BACTEC MGIT-960 culture. The 10 positive samples were further subjected to drugs susceptibility testing and line probes assay test to find out rifampicin, isoniazid, streptomycin and ethambutol resistant and it was found that 6 M.TB isolates were resistant while 4 were sensitive to rifampicin and isoniazid. Among the 6 resistant M.TB strains, 4 showed mutation in rpoB gene at 531, 516 and 526 codons.
Conclusion: Majority of MDR-TB patients' attendants had drug-resistant tuberculosis and the rate of drug susceptible TB was low.

Keywords: Assay, Multi-drug resistant tuberculosis, Sputum, Tuberculosis.

INTRODUCTION

Tuberculosis is the most common cause of death among women in the developing countries and is ranked 7th in the list of life threatening disease in the world. WHO (World Health Organization) reported that Pakistan ranks 6th by number of tuberculosis cases in the world[1]. Multi-drug resistant tuberculosis (MDR-TB) caused by Mycobacterium tuberculosis (resistant to isoniazid (INH) and rifampicin (RIF) is a sever threat to the ongoing TB-eradication program around the globe. More than 450,000 MDR-TB cases are expected to occur every year in the world[1,2]. MDR-TB was recognized after the introduction of anti-TB drugs, with the description of streptomycin (STR) resistance[3,4]. MDR-TB is characterized by resistance to INH and RIF[5]. M.TB becomes resistant to INH, rifampicin (RIF), ethambutol (EMB) and STR due to mutations in several genes in the genome of Mycobacterium Tuberculosis[6]. M.TB becomes resistant to RIF due to the mutations in rpoB gene coding the beta subunit of RNA polymerase. More than 90% of the mutations have been found in 81 bp (507 to 533 codons) core region of RNA polymerase beta subunit of rpoB gene[6,7]. Riordan et al, recommended line probe assay (LPA) for the detection of MDR-TB in 2008[8]. Culture is considered the current diagnostic gold standard and is essential for detection of smear microscopy negative cases. The limit of detection is considered to be 100 bacteria/ml of sputum sample[9]. Eastern Mediterranean region constitutes 6% of overall global TB and 4.3% of MDR-TB cases. Prevalence of MDR-TB in this region is 3.3% of all tuberculosis cases out of which, 50% cases occur in Pakistan[10]. It is also reported that the detection rate of MDR-TB cases is relatively low than the actual prevalence of the

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disease. The low case detection rate observed in the Eastern Mediterranean region is mainly because of low detection rate in Pakistan and Afghanistan. If this remains unexplored, then a major outbreak of TB can be expected in Pakistan in the coming years[10,11]. The rate of MDR-TB in Pakistan varies from 2.3% in untreated individuals to an alarming 17.9% in individuals who have been previously treated for the disease. Several studies have documented a high prevalence of MDR-TB in Pakistan[12-14]. A recent study has shown a constant increase in the number of MDR-TB cases from 1990-2007, more than 15,000 cases reported during the study period[15]. So far no study has been conducted to study the occurrence of MDR-TB in MDR-TB patients' attendants in North Western Pakistan. The current study was therefore conducted to determine the occurrence and drugs susceptibility pattern in MDR-TB patients' attendants in North Western Pakistan.

MATERIAL AND METHODS

This cross sectional study was carried out at Peshawar Tuberculosis Research Laboratory, Provincial TB Control Program Hayatabad Medical Complex Peshawar, from August 2013 to March 2014. Data and sputum samples were collected from MDR-TB patients' attendant in MDR-TB center located in Lady Reading Hospital Peshawar. The scientific and ethical committee of the Lady Reading Hospital Peshawar-Pakistan approved this Research work.

Sample size was calculated with the help of WHO sample size calculator, the confidence level was kept at 95%, expected population proportion 0.843 and absolute precision required 9%. The sampling technique used was non-probability purposive sampling. Based on these parameters the sample size calculated was 480 specimens.

Male and female population of the specific age range from 08-65 years, peoples who belong to Peshawar division, were patients family member and were in closed contact with the MDR-TB patients' were included in this study while people having age less than 08 years or higher than 65 years and those who were having any co-infection reported or provided no history of it, were excluded from this study. These 480 sputum samples were digested and decontaminated with N-acetyl-L-cysteine and NaOH. Smear was prepared from the sputum sample over a microscopic slide with the help of sterile wire loop and observed under oil immersion for the presence of mycobacteria after Ziehl-Neelsen (ZN) staining.

The processed sputum samples were inoculated on "Lowenstein Jensen (LJ) culture" medium for 2 months and mycobacteria growth indicator "BACTEC MGIT-960 culture" for 4 to 21 days for the growth of M.TB. Confirmation of positive growth was done by Nitrate reductase

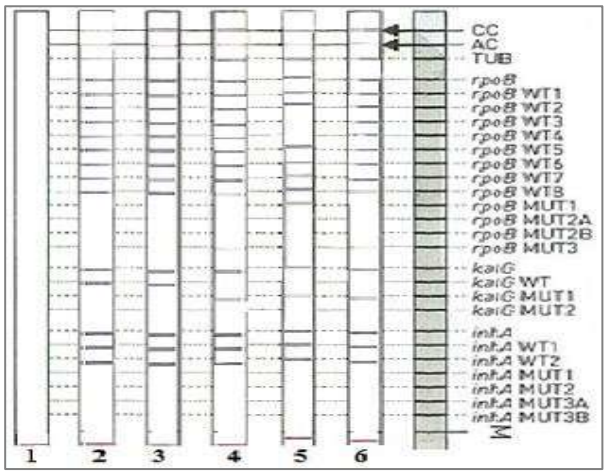

Figure: Representative DNA patterns of M. tuberculosis showing resistant and sensitive pattern through LPA.

test. Drug susceptibility testing (DST) was performed by inoculating the media with a pure growth culture. The critical concentration of drugs solution was added into each of the labelled BACTEC MGIT-960 tubes containing the culture media, 1.0µg, 0.1µg, 1.0µg, 5.0µg, 25µg for STR, INH, RIF, EMB and pyrazinamide, respectively. DNA extracted from culture positive isolates, was subjected to 30 PCR cycles for amplification. Initial denaturation was done at 95°C for 15 min, 10 cycles of denaturation were done at 95°C for 30 sec and initial elongation was done at 58°C for 120 sec, 20 cycles were done for

final denaturation at 95°C for 25 sec, annealing at 53°C for 40 sec, elongation at 70°C for 40 sec, Final elongation was done at 70°C for 8 min. The amplified tubes were removed from the PCR for hybridization. For hybridization process, Strips were used, the double stranded DNA amplicons were denatured to single strands in TwinCubator by adding 20 µl of denaturing buffer and 20 µl of the DNA amplicons followed by One ml stringent wash buffer, 1 ml Rinse solution and one ml of steriled distilled water. The developed strips were dried and transferred to the Genotype MTBDR plus score sheet to examine the M.TB target DNA sequences associated with INH and RIF resistance. The results were analyzed by Graphpad Prism version 5.0. percentages and frequency were calculated for different variables.

RESULTS

Out of 480 sputum samples, 6 were positive through ZN staining. The sputum samples were further incubated on different culture media for 8 weeks at 37°C and it was found that, 10 (2.08%) samples were positive for M.TB as shown in table-I.

All the 10 positive cultural growths were further subjected to LPA test for the detection of INH and RIF drugs resistant and it was found that 6 (2.43%) participants were MDR-TB and 4 (0.81%) were drugs sensitive. Banding patterns of oligonucleotides are shown from left to right direction as mentioned in figure.

All the 10 M.TB cultural growths were further subjected to BACTEC MGIT-960 tubes for INH, RIF, STR, ETH and PZA susceptibility testing and it was found that 6 isolates were RIF and INH resistant (MDR-TB) while 4 M.TB isolates were sensitive to INH and RIF drugs. Similarly, STR, ETH and PZA drugs patterns are also given in table-II.

Out of total number of participants, 40 (8.33%) were with history of smoking whereas 440 (91.66%) participants declared to be non-smoker. Amongst the smokers, 2 participants were MDR-TB. In 234 rural participants, 2 (0.85%) were drugs sensitive and the remaining 232 were negative for TB. In 8 treated persons, 2 (25%) were reported MDR-TB following LPA and DST.

Table-I: Frequency of mycobacterium tuberculosis in MDR-TB patients' attendants reported through ZN staining, LJ and MGIT-960 culture.

Tests	Total Participants	Positive	Percentage
ZN staining	480	6	1.25
LJ culture	480	10	2.08
MGIT-960 culture	480	10	2.08

Table-II: Representative patterns of drugs resistant and drugs sensitive M. tuberculosis reported through drugs susceptibility testing.

Positive Samples	Drugs Susceptibility Testing				
Drugs	INH	RIF	STR	ETH	PZA
1	R	R	S	S	R
2	R	R	R	S	R
3	R	R	R	R	R
4	S	S	S	S	S
5	S	S	S	S	S
6	R	R	S	S	R
7	R	R	R	R	R
8	R	R	R	R	R
9	S	S	S	S	S
10	S	S	S	S	S

R=resistant, S=Sensitive

In 236 non-treated candidates, 4 were Sensitive to INH and RIF while the remaining 4 were MDR-TB. Among the urban samples, 3.25% (8) participants were positive for M.TB following growth on BACTEC-MGIT-960 culture. The study participants were categorized into different groups. Group 1 declared monthly income of ≥5000 Pakistani rupees whereas monthly income of group 2 was ≤6000. In Group 1, 4 (2.77%) cases were reported MDR-TB and in group 2, only 2 (0.59%) candidates were MDR-TB. Similarly, 124 participants, having age 5-20 years were included in group 1 and 180 above 20 years were included in group 2. In group 1, only 4 (3.22%) were MDR-TB and 2 (1.11%) participant were reported MDR-TB in group 2. The overall prevalence of MDR-TB was 1.25% in our current study as shown in table-III.

Table-III: Percentage distribution of M. tuberculosis and MDR-TB on the basis of different factors.

		LJ & MGIT culture		Line probe assay			
Actors			%	**Sensitive to INH & RIF**	%	**MDR-TB**	%
Rural			3.25	2	0.81	6	2.43
Urban	234	2	0.85	2	0.85	0	0
Smoker	40	2	5	0	0	2	5
Non-Smoker	440	8	1.81	4	0.9	4	0.9
Medicated participants	8	2	25	0	0	2	25
Non - Medicated participants	472	8	1.69	4	0.84	4	0.84
Income/month (≥5000)	144	4	2.77	0	0	4	2.77
Income/month (≤6000)	336	6	1.78	4	1.19	2	0.59
Group 1 (5-20)	124	4	3.22	0	0	4	3.22
Group 2 (21-36)	180	6	3.33	4	2.22	2	1.11
Total	480	10	2.08	4	0.83	6	1.25

DISCUSSION

In Pakistan, the National Directly Observed Treatment, Short course (DOTS) program aims to provide early diagnosis and treatment for MDR-TB patients and is planning to cover the entire country in a phased manner. Early identification and treatment of tuberculosis patients may reduce morbidity and mortality rate in the community[16]. Patients' attendant of MDR-TB are at high risk of developing TB and MDR-TB. In our study, 1.2% positive sputum samples were reported following ZN staining. The results of current study are in agreement with another finding, who detected 1.1% pulmonary TB prevalence among newly diagnosed patients[17]. Siddiqui et al[18] found 15% positive cases following LJ and BACTEC-MGIT-960 media culture in Faisalabad-Pakistan. Another report on different TB diagnostic techniques compared positive culture growth of M.TB on solid LJ culture and BACTEC-MGIT-960 culture. Out of 527 sputum samples, 428 (81%) positive cases were reported by BACTEC- MGIT-960 culture and 411 (78%) through LJ cultures media[19]. Andrea et al[20] reported 9.4% resistant cases to one anti-TB drug and 15% were MDR-TB cases. Another report on MDR-TB patients' at Holy Family Hospital, Rawalpindi-Pakistan to find out the DST for M.TB and they recorded high resistant rate for STR, EMB and PZA[21]. Further research work was carried out on TB disease to determine the frequency of TB among MDR-TB

contacts[22]. Out of 2112 MDR-TB contacts, 108 (5%) developed TB. Our results are supported by Siddiqui et al[18] who reported 5% positive cases through ZN staining in suspected TB patients in Faisalabad-Pakistan. In our study, the prevalence of M.TB in 8 previously treated and 472 were non-treated participants for TB disease. In treated 2 (25%) were positive for TB and they were MDR-TB cases. Among 472 non-treated, 8 participants were positive for TB of which 4 (1.69%) were MDR-TB and 4 (0.84%) were drugs sensitive cases. Similar study was performed in Rawalpindi-Pakistan. Out of total 30 studied cases, 83.3% had previously taken anti-TB drugs[21]. The positive growth cultures reported during current study were subjected to DST and it was found that 6 cultures were resistant to INH, RIF. In these 6 MDR-TB isolates, 4 (3.22%) were reported in group-1 having age (5-20 years) and 2 (1.11%) were reported in group-2 (21-36 years). Similarly, the prevalence of MDR-TB in our studied participants with monthly income (≥5000) was 4 (2.77%). In another survey, 286 cases were studied in Pakistan of which 36 were resistant to at least one drug and 37 isolates were resistant to INH, 15 to STR, 13 to EMB, 4 to RIF and 30 to PZA. In total patients, 88.7% were under the age of 40 years while 9.4% had income was less than 100$/month[23]. Among the six M.TB resistant strains reported, the resistant pattern were located at the point of rpoBMUT3 and KatGMUT1 mutation (RIF and INH resistant), rpoBMUT1 and KatGMUT1 mutation and rpoBMUT1 and KatGMUT1 mutation, 4 showed mutation in rpoB gene at 531, 516 and 526 codons and 2 MDR-TB strain showed mutation at KatG gene. Another study examined 108 smear positive pulmonary tuberculosis samples through LPA and reported the M.TB resistance rate, the M.TB resistance rate were 92.5%, for RIF and 76.3% for INH[24]. Mutations at 531 and 533 codons of rpoB gene were detected. Mutation of katG gene was detected in 55.9% and inhA promoter mutations were reported in 11.9% of INH resistant isolates at the laboratory of Aga Khan University Karachi-Pakistan[24].

CONCLUSION

Majority of MDR-TB patients' attendants had drug-resistant tuberculosis and the rate of drug susceptible TB was low.

RECOMMENDATION

This study highlight the important of early detection of TB and MDR-TB in MDR-TB patients' attendants, who represent a high-risk group. Molecular based detection methods offer a great chance to improve early diagnosis and treatment of these cases which will also reduce morbidity, mortality and transmission of infection in the community.

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CONFLICT OF INTEREST

This study has no conflict of interest to declare by any author.

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