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LINE PROBE ASSAY, THE BEST MOLECULAR DIAGNOSTIC TOOL, AND IMPROVE THE MULTI DRUG RESISTANCE DETECTION IN PULMONARY TUBERCULOSIS CASES IN BALOCHISTAN



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Abstract

Mycobacterium tuberculosis (MTB) appears resistant to first line antibiotics e.g. rifampicin and isoniazid is said to be Multi-drug resistant Tuberculosis (MDR-TB), Globally 15.3 million cases emerge where as in Pakistan 1.8 million cases reported included Pakistan in 22 high burden cases. For MDR-TB detection in this study Line Probe Assay (LPA) a molecular technique was used. 400 patients were studied in this study after sample collection in open space for pulmonary sample and for extra pulmonary samples were taken in sterile syringe or containers auramine staining was performed and examine under 40X microscope, the DNA was extracted by Genolyse Method. The master mix was adjusted by mixing of master mix A and mix B for further uses. e.g. Amplification and hybridization: for amplification process thermo-cycler was used as PCR amplification and for hybridization the PCR products were analyzed to the strips on which probe are presents and interpreted.

The study was conducted among different ethnic groups and both male & female genders of Balochistan. Overall 400 smear negative and positive samples from TB DOTS and PMDTS were applied on LPA for the diagnosis of Mono and MDR-TB. In total 400 cases, 236 (59%) were MTB positive, 162 (40%) were found MTB negative and 2 were detected as invalid. In this study analysis was also based on gender, ethnicity and site (DOTS and PMDTS).

In conclusion MDR-TB detection by LPA is more sensitive technique to others and prevalence rate of MDR-TB in enrolled patients at PMDTS and DOTS program at alarming stage, serious threats to public. The outcomes of this study enhance about the mutation occurs in MTB is highly which promote TB in MDR-TB and in future MDR-TB in to XDR-TB. It is highly suggested for the development in the diagnostic techniques and tools .i.e. LPA for MDR-TB detection method.

Keywords: Multi Drug Resistance, Line probe assay, pulmonary tuberculosis, extra pulmonary tuberculosis

INTRODUCTION

Multi-drug Resistant Tuberculosis (MDR-TB) means *Mycobacterium tuberculosis bacilli* (MTB) shows resistant to first line antibiotics e.g. rifampicin and isoniazid. This problem occurred worldwide, an estimation of WHO more than 15.3 million new cases of MDR emerged globally, where as 13 million patients have been diagnosed and 2.4 million patients died in 2016 (1). In Pakistan 1.8% of MDR-TB is reported from new TB cases which is alarming sign for whole population, and included 22 high burden MDR-TB countries (2). The exiting statistics indicated that TB will be replaced by MDR-TB cases, so transmission is not the only source but poor treatment and unawareness about TB also promote MDR-TB cases (3, 4).

The basic and primary method for TB screening is smear microscopy, with the less than 70% detection of case rate (5, 6). In 2010 out of 5.8 million 2 million (34.4%) TB cases were misdiagnosed that were to be found smear negative worldwide (7). In past decade, conventional DST (drug susceptibility testing) which based on solid media (a gold standard for TB diagnosis) was done for detection of drug resistant *Mycobacterium tuberculosis* (MTB), the method was long lasting and consume more time as one to two months. Subsequently the use of DST, a liquid-based media, helped to reduce all the limitations of results time. To minimize these limitation of liquid-based media another new technique was introduced Known as Line probe assay (LPA). The World Health Organization (WHO) permitted and specialized LPA in 2008 as a molecular method for the fast and accurate diagnosis of MTB and MDR TB, this technique shows in real-time detection of rifampicin (RMP) and isoniazid (INH) resistance and the results in some hours with good accuracy rate as compares to other methods (8).

Line probe assay is a Rapid molecular technology used for the diagnosis of rifampicin and isoniazid resistance in MTB which detect mutations on genetic level. When mutation occurs in the *rpoB* gene of RNA polymerase (encoding-subunits) produces rifampicin resistance of 95-100% (9). Whereas *katG* & *inhA* genes are mutated then isoniazid resistance produced, codon 315 of *katG*, and promoter region of *inhA* mutation have more 65-90% contribution in Isoniazid resistance (10).

The forward primer of *rpoB* (81 pair hyper-variable region) is 5-CGACCACTTCGGCAACCG-3, where its reverse primer is 3-TCGATCGGGCACATCCGG-5, the gene encoded the Isoniazid are *KatG* (codon 315) with forward and reverse primers 5-TCGGCGGTCACACTTTCGGTAAGA-3 and 3-GCGACGCGTGATCCGCTCATAG-5 and the other gene is *inhA* (promoter region) with the forward primer 5-CGAGCGTAACCCAGTGCAGAAAGT-3 and its reverse primer is 3-CCCCGGTGAGGTTGGCGTTGAT-5, these genes are responsible for the mutation of bacterial DNA to show drug resistance (21, 22). However, smear-positive, smear-negative sputum and scanty positive cases has been evaluated by LPA to detect drug resistance in all these kind of specimens. But in high-grade smear-positive samples MDR TB is very easy to detected (11, 12).

The current study designed to explore the diagnostic achievements of LPA to detect MDR TB in Pulmonary and extra pulmonary TB cases. For MDR TB samples, the specificity and sensitivity of LPA is more accurate, and high, we also examine mono-DR-TB and Multi-DR-TB on the gender and ethnicity based in Balochistan.

MATERIAL AND METHODS

STUDY POPULATION

Total 400 patient were studied in this study who were recommended by their physicians as suspicion of TB and MDR-TB from September 2017 to November 2018. These may include the mistreated, return after default, relapse and failure in other word all were smear positive samples. And smear negative cases were also added, some of new case from TB DOTS were also added in the present study.

Sputum samples were collected in open space and then carried in ideal containers which were clean, dry, leak proof, wide-necked, and pure sputum was prepared as a smear by using sterile bamboo sticks, dried for 15 minutes to spread smear evenly, and hot fixation at 85C for 3 minutes (14-16). Then applied auramine O staining, 0.1% auramine solution was used over the smear for 15 minutes, decolorizer 0.5% HCL for 3 minutes, and 0.3% methylene blue the counter stain for 1 minute (15-16). At last, smear was dried for half an hour; the prepared slides were inspected under LED fluorescent microscope on 40X microscope.

For line probe assay following steps were performed,

DNA EXTRACTION

After decontamination of all samples, the DNA was extracted by Genolyse Method. A 500 ul of pallet was transferred into DNA extraction tube and centrifuged for 15 minutes at 10000 rpm. The supernatant was discarded and 100 ml of lysis buffer (A-LYS) was added over the pallet and vortexed for 15 seconds. The tubes were placed in water bath for 5 minutes at 95° and spun down lysate. Neutralization buffer (A-NB), with a volume of 100 ml, was added over lysate and vortexed for 4 to 5 seconds. Then centrifuged the neutralized lysate at 10000 rpm for 5 minutes. Finally, eppendorf tube was used to store supernatant (DNA), from where 5 ul was used for PCR amplification (17, 18).

MASTER MIX PREPARATION

Mix A (AM-A GT MTBDRplus VER 2.0) contains buffer solutions, DNA nucleotides, enzymes like Taq polymerase and mix B (AM-B GT MTBDRplus VER 2.0) contains alkalis, exact primers and colure pigments to use these both mix is called master mix (19, 20). 10 ml of reagent-A and 35 ml of reagent-B required for one complete PCR Batch (18).

AMPLIFICATION AND HYBRIDIZATION

A volume of 5 ul of extracted DNA solution and 45 ul of master mix were mixed well in tube, and then tubes were placed into the thermo cyler for amplification.

According to the manufacturers' directions (Genotype MTBDR test) was carried out for using the reagents presented in the kits. Detection and analysis of the results were visibly labeled in shape of bands for instruction (18, 21, 22). Then the results were interpreted with the manufacturer manual to describe resistance or sensitivity. A positive *M. tuberculosis* control (TUB) band indicated the presence of M tuberculosis in the sputum and non-sputum samples Line Probe Assay was carried out only on the smear-positive samples.

RESULTS

Present study was conducted among different ethnic groups, both male & female genders of Balochistan region of Pakistan. Overall 400 smear negative and positive samples from TB DOTS and PMDTS were used for LPA to diagnose Mono and MDR-TB. Among these 400 cases, 236 (59%) were MTB positive, 162 (40%) were found MTB negative and 2 were detected as invalid.

Among 236 MTB positive cases, Mono-DR and MDR-TB were found as 44 (19%) and 69 (29%), respectively. Whereas, among 195 total male patients, 19 (17%) were mono-DR and 38 (33%) were MDR-TB and among all these 58 (50%) cases were found to be sensitive to first line LPA drugs. On the other hand, among 205 total female patients, 25 (21%) were mono-DR and 31 (26%) were MDR-TB. whereas and among all these, 65(53%) cases showed sensitivity to first line LPA drugs as shown in Table I.

Table I. Gender based TB & MDR-TB detection in Balochistan region of Pakistan

Gender wise Groups	Total Cases	% age	MTB Positive	% age	Mono-DR	% age	MDR	% age	Total Sensitive	% age	Total Negative	% age	Total Invalid
Male	195	49	115	59	19	17	38	33	58	50	79	40	01
Female	205	51	121	59	25	21	31	26	65	53	83	40	01
Total	400	100	236	59	44	19	69	29	123	52	162	40	02

Among various ethnic groups (Pashtoon, Baloch, Hazara, Settlers) studied from Balochistan region of Pakistan, overall 44 Mono-DR and 69 MDR-TB cases were observed. Among all positive cases, Pashtoons were on top with 188 (47%) total cases where 19 (16%) were Mono-DR, 32 (26%) were MDR-TB and 70 (58%) were MTBDR plus sensitive followed by 113 Baloch had 10 (16%) Mono-DR and 21 (33%) MDR-TB with 33 (51%) sensitive cases. Moreover, Hazaras and Settlers include total patients 56 and 43 with 14% and 11%. Among total Hazara community patients, 9 (29%) were Mono-DR and 9 (29%) were MDR-TB with 13 (42%) sensitive cases whereas in total Settlers, 6 (30%) were Mono-DR and 7(35%) were MDR-TB patients having 07(35%) dMTBDRplus sensitive cases as illustrated in Table II.

In overall 400 cases, n=293 (73%) were taken from TB DOTS however n=107 (27%) cases belonged to PMDTS in Balochistan. wherein all TB DOTS cases, n=131 (45%) were MTB positive where n=06 (05%) were Mono-DR, n=02 (02%) were MDR-TB and n=123

(94%) were MTBDRplus sensitive. Whereas, in total PMDTS cases, n=105 (98%) were MTB positive where n=38 (36%) were Mono-DR, n=67 (64%) were MDR-TB as mentioned in Table- III.

Table II: Ethnicity Based TB & MDR TB Detection in Balochistan

Ethnic Groups	Total Cases	MTB Positive		Mono-DR		MDR		Total Sensitive		Total Negative		Total Invalid	
		% age	% age	% age	% age	% age	% age	% age	% age				
Pashtoon	188	47	121	64	19	16	32	26	70	58	65	35	02
Baloch	113	28	64	56	10	16	21	33	33	51	49	44	-
Hazara	56	14	31	55	9	29	9	29	13	42	25	45	-
Settlers	43	11	20	47	6	30	7	35	07	35	23	53	-
Total	400	100	236	59	44	19	69	29	123	52	162	40	02

Table III. TB & MDR TB Detection rates from TB DOTS & PMDTS cases

Site	Total Cases	MTB Positive		Mono-DR		MDR		Total Sensitive		Total Negative		Total Invalid	
		% age	% age	% age	% age	% age	% age	% age	% age				
TB DOTS	293	73	131	45	06	05	02	02	123	94	162	55	0
PMDTS	107	27	105	98	38	36	67	64	0	0	0	0	02
Total	400	100	236	59	44	19	69	29	123	52	162	40	02

DISCUSSION

This study was performed in Balochistan where no such study was conducted earlier for the diagnosis of Mono and MDR-TB by LPA MTBDRplus assay which is a sensitive and rapid test for the diagnosis of drug resistant TB based on PCR and hybridization.

In previous decade both, patient management and infection control departments, were playing crucial role for MDR-TB positive cases detection (23). In retreatment cases MDR-TB prevalence reached at high risk level due its mortality rate is about more than 65% in Pakistan's and few drugs has so far been effective against TB to control this threat (24-26). According to an estimation of WHO in 2010 report, the percentage of retreatment cases was 28% (2200 out of 7795 cases) which shocked the whole world (27). This significant boost in the worldwide prevalence of MTB cases resistant to drugs elevated, more fast and effective drug resistant MTB detection technique for the commencement of proper and early doctoring of the patients and then TB control program manage new techniques to decrease this problem (28). Due to this reason, in this study an attempt has been made to launch the molecular diagnostic method Line Probe Assay (LPA) to detect the mutated genes subjected for the resistance of drugs and this method is also a rapid diagnostic method. This fast and effective diagnostic method for the daily basis detection

of MDR-TB made it possible to decline the TB and MDR-TB from the population and provide proper treatment on proper time.

The outcome of this current study shows that rapid molecular method was more consistent than other conventional DST method based on culture. Line probe assay have surpassing sensitivity for mono-DR and multi-DR finding rate to prove this fact. The technique could be most effective to detect the *rpoB* gene, accountable gene for RIF resistance with 99.5% which is more than conventional DST. Previous studies also proved that LPA findings are more accurate than other conventional methods (29, 30). For INH resistance detection Line Probe Assay performed same uniformity as well as MDR-TB and for INH resistance to detect two mutated genes in *hA* and *katG* with 99.5% accurate sensitivity rate. The INH resistance detection was reported in the previous studies with significant variation (31, 32). Moreover, line probe assay is much effective technique for the detection of both mono and multi drug resistance TB and its performance is much explorative than other detective techniques.

In this study 236 out of 400 cases were detected as TB positive where 69 cases were MDR-TB and 44 were mono-DR TB by LPA which is an accurate technique for genotypic DST. LPA favors both health management and patients. So, to overcome this problem LPA is the best diagnostic opportunity. The previous study also proves the short turnaround time 24-48 to detect MDR-TB and on same occasion show the mutated genes responsible for drug resistance (33). Generally, to overcome the MDR-TB, line Probe Assay must be performed for diagnosis like as its drugs for the treatment, moreover, this technique is 50% less cost effective than other conventional DST methods (22). Seeing to this value, rapidity and low cost effectiveness and to stop the conversion of MDR-TB to XDR-TB (extensive drug resistance mean shows resistance to the second line of anti TB drugs) by means of rapid detection, so, the molecular approaches for the detection of MDR-TB must be promoted as routine TB diagnosis centers to control MDR-TB cases.

CONCLUSION

MDR-TB detection by LPA is more sensitive technique to others and prevalence rate of MDR-TB in enrolled patients at PMDTS and DOTS program at alarming stage, a serious threat to public. To overcome this issue, molecular LPA should be compulsory for the MTB diagnosis on routine bases and to make possible reduction of MDR-TB cases. The outcomes of this study enhance about the mutation occurs in MTB is highly which promote TB in MDR-TB and in future MDR-TB in to XDR-TB. It is highly suggested for the development in the diagnostic techniques and tools .i.e. LPA for MDR-TB detection.

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