Multiplex PCR for Rapid Diagnosis of Drug Resistant *Mycobacterium Tuberculosis*

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ABSTRACT

Objective: To evaluate a multiplex PCR for rapid diagnosis of drug resistant *mycobacterium tuberculosis* (MTB) strain.
Study Design: Cross-sectional observational study.
Place and Duration of Study: Department of Microbiology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, from January to September 2018.
Methodology: Over a period of 8 months, a total of 84 cultured positive samples were included in the study using non-probability sampling techniques. MTB isolates were phenotypically characterised using MGIT 960 system for anti-tuberculosis agents including rifampicin (RIF), isoniazid (INH), ethambutol (EMB) and Streptomycin. The DNA was extracted using Gentra system DNA extraction kit. The multiplex PCR was optimised for genetic characterisation of MTB samples for rpo B (rifampicin), kat G (isoniazid) and emb B (ethambutol) gene. The gel electrophoresis was performed to observe comparative banding pattern of amplified gene products.
Results: For detecting drug resistance, the specificity and sensitivity of multiplex PCR in isolates was 100% and 100% for rifampicin, 100% and 71% for isoniazid, and 100% and 60% for ethambutol, respectively. When compared to phenotypically resistance results, the positive predictive value (PPV) was 100% each and the negative predictive value (NPV) was calculated to be 100%, 74% and 71% for RIF, INH and EMB, respectively.
Conclusion: Multiplex PCR is a useful gadget for quick determination of drug-resistant TB in specimens, hence permitting an initial therapeutic approach. However, for accurate management of patients, phenotypic method should be used to confirm results.

Key Words: *Multiplex PCR, Ethambutol, Rifampicin, Tuberculosis, Drug resistance.*

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INTRODUCTION

Tuberculosis is one of the major global health problem due to evolution and spread of drug resistant MTB.[1] According to WHO, Pakistan ranked fourth in MDR-TB.[2] Success of TB control effort is highly dependent on rapid diagnosis and drug susceptibility determination of MTB strains. However, it takes weeks to get phenotypic results due to slow growth rate of bacteria. Nucleic acid amplification test (NAAT) are promising for a rapid diagnosis of DR-TB strains.[3] The MTB strains are resistant to first line anti-TB drugs such as isoniazid (INH) and rifampicin (RIF) are denoted as MDR-MTB.[4] The MDR-MTB strains resistant to fluoroquinolones and any one of second line injectable agents, *e.g.* amikacin, kanamycin, capreomycine are XDR-MTB.[5] Although various methods are available to diagnose MTB,[6] ZN staining and culture that remains gold standard. The microscopy of acid fast bacilli by ZN staining is easy and simple. However, it is subjective and cannot detect MTB at low quantity. The gold standard of MTB is culture. It has high sensitivity but time consuming and requires further delay for susceptibility testing.[7] The molecular methods are cost effective, rapid, and reliable with high sensitivity and specificity than phenotypic methods.[8]

Mutation in the rpo B gene, that encodes the beta sub-unit of RNA polymerase, are responsible for rifampicin resistance.[9] The mutations in *kat G, inh A,* and ory *R-ahpc* genes accounts for the resistance against isoniazid.[10] Ethambutol (EMB) inhibits arbinosyl transferees and mutation in emb A , emb B and emb nce C genes produce resistance against this agent.[11] Furthermore, mutation in gyr A and gyr B genes confer resistance to fluoro-quinolones.[12,13] Molecular methods like PCR is cost effective with high sensitivity and specificity. Gene Xpert MTB/rifampicin assay has been endorsed by WHO for the diagnosis of pulmonary and extra pulmonary TB in endemic countries with 98% sensitivity and specificity.[14,15] However, it detects resistance against rifampicin only.[16] However, multiplex PCR differ from conventional PCR

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because we use multiple primer simultaneously to detect multiple MTB genes by setting optimum conditions in multiplex PCR. Therefore, in this study, the aim was to optimise a multiplex PCR method for diagnosis of mutations associate with resistance to RIF, INH and EMB in DR-MTB cultured isolates.

METHODOLOGY

This study was conducted, Department of Microbiology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, from January to September 2018. The study was ethically approved by Institutional Ethical Committee. A total of 84 samples were collected from MTB repository at AFIP. All smear positive pulmonary and extra-pulmonary drug resistant MTB samples were included in the study. Anti-TB drug sensitive MTB samples were used as control. The repeated sample of same patient and *mycobacterium* other than *tuberculosis* (MOTS) were excluded. Smear positive specimens were treated before with standard sodium hydroxide-N-acetyl-L-cysteine method for decontamination, digestion, homogenisation and concentration. A smear was prepared and stained with Ziehl-Neelsen´s stain. In this procedure, smear positive specimen was inoculated in BACTEC MGIT 960 system to culture MTB isolate. Culture media (7H9 broth, 7mL) was inoculated in bar-coded mycobacterial growth indicator tube (MGIT). Growth supplement was added for enhancing growth of MTB and an antimicrobial mixture PANTA was added to MGIT tubes to suppress contamination. When an inoculated specimen yielded positive culture in MGIT system, we confirmed it by ZN staining and by MGIT 960 for TB identification test, which is a rapid chromatographic immunoassay for MTB antigen detection. Setting up for direct DST, lyophilized PANTA was reconstituted with 15Ml of SIRE supplement (Becton Dickinson Diagnostic Systems, Sparks, MD) and mixed thoroughly. Lyophilized drugs (same as those used in MGIT) INH and RIF were reconstituted with 4 mL of sterile deionized (DI) water and mixed well.

A set of three MGIT tubes was prepared per specimen for performing the direct DST. One tube was labelled as GC, one for INH and the other for RIF. PANTA-SIRE supplement mixture (800 μL) was added in the labelled MGIT tubes. Then, the respective drugs were added in labelled tubes. The GC, INH and RIF tubes were placed in the set carrier and it was entered in the instrument. The first tube in the set carrier was always the GC tube. The growth unit (GU) value $\geq$400 for GC indicated the completion of test. Two hundred and fifty μL of cell lysis solution was added to the 1.5/μL eppendorf tube containing 50/μL of MTB culture isolates and incubated at 65°C for 15 minutes after thorough mixing with pipette.

After cell lysis, samples were cooled at the room temperature and then 100/μL protein precipitation solution was added to cell lysate followed by vortex. After protein precipitation step, the supernatant containing DNA was transferred carefully into eppendorf tube containing 250/μl isopropanol (100%) and mixed by inversion. The samples were centrifuged again at 15000 g for 5 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol and centrifuged at 15000 g for 15 minutes. The ethanol was removed without disturbing the DNA pellet using pipette. The pellet was dried by inverting tubes on absorbent paper followed by air dry for 15 minutes. The DNA pellet was hydrated in 10 μL of DNA hydration solution and incubated at 65°C for 1 hour. The DNA samples were then stored at -80°C. Described primers were used to amplify *rpo B, kat G* and *emb B* genes as follow;[17,18] forward 5'- CGGCGATGAGCGTTACAC-3'and reverse 5'- CGTCCTTGGCGGTGTATT-3' for *kat G* with 458bp PCR product, also forward 5'-CAGACGTTGATCAACATCCG-3' and reverse 5- TACGGCGTTTCGATGAAC-3' for *rpoB* gene with 305bp PCR product forward primer for *emb B* gene was 5'- GGATGCCGTTCAACAACGG-3' and reverse 5'- CGCGAACCCTGGTGGCTTC-3' with 306bp PCR product.

Multiplex PCR were optimised and done in a final volume of 27 μl for the amplification of the *rpo B, kat G* and *emb B* genes as mentioned in Table II. The thermo cycler programme of PCR reaction mixture was including an initial denaturation step of 5 minutes at 95°C; 40 cycles of 30 seconds at 95°C, 30 seconds at annealing temperature of 57°C and 45 seconds at 72°C and final extension step at 72°C for seven minutes. The PCR amplified product was separated on 1% agarose gel and visualised under ultraviolet light.

Results were subjected for statistical analysis on SPSS version 24. Descriptive statistics including sensitivity, specificity, negative predictive value, positive predictive value and diagnostic accuracy of phenotypically resistant isolates of MTB and drug sensitive samples as a negative control for multiplex PCR.

RESULTS

Among 84 MTB isolates, MGIT culture method detected 18 (21%) isolates as MDR, and 66 (79%) as single drug resistant including 17 (20%) RIF resistant, 39 (47%) INH resistant and 10 (12%) EMB resistant isolates. Out of the 18 MDR MGIT culture positive isolates, only 9 (50%) showed all two mutated bands on multiplex PCR. Moreover, among single drug-resistant, *i.e.* 17 RIF, 39 INH and 10 EMB MGIT culture positive isolates, mutated bands were detected in 17 (100%) of *rpo B* gene for RIF, 28 (71%) of *kat G* gene for INH and 6 (60%) of *emb B* gene for EMB in multiplex PCR. The characteristic banding pattern obtained by using the multiplex PCR for RIF, INH and EMB resistant isolates is shown in Figures 1 and 2. Sensitivity, specificity, positive predictive value, negative predictive value, diagnostic accuracy of

Table I: The primers and PCR conditions for genotypic characterisation of MTB samples.

Anti-TB drugs	Targets site	Primers	Tm (°C)	PS	Ref
Rifampicin	*rop B* (531)	F: CAGACGTTGATCAACATCCG R: TACGGCGTTTCGATGAAC	57	305	17
Isoniazid	*kat G* (315)	F: CGGCGATGAGCGTTACAC R: CGTCCTTGGCGGTGTATT	57	458	17
Ethambutol	*emb B* (345)	F: GGATGCCGTTCAACAACGG R: CGCGAACCCTGGTGGCTTC	57	306	18

Tm = Temperature; PS = Product size; Ref = Reference

Table II: Multiplex PCR reaction mixture.

Reagents	Concentration	Volume
Distilled water		16.1 µl
Forward primers for *rpo B, kat G* and *em B* genes	1 µM	3 µl
Reverse primers for *rpo B, kat G* and *emb B* genes	1 µM	3 µl
Magnesium chloride (MgC12)	2.5 µM	2.5 µl
Buffer without MgC12	2 µM	2 µl
dNTPS	0.5 µM	0.5 µl
Taq polymerase	0.5 µM	0.5 µl
DNA	1-5 ng	1.4 µl
Total	1-5 ng	25-27 µl

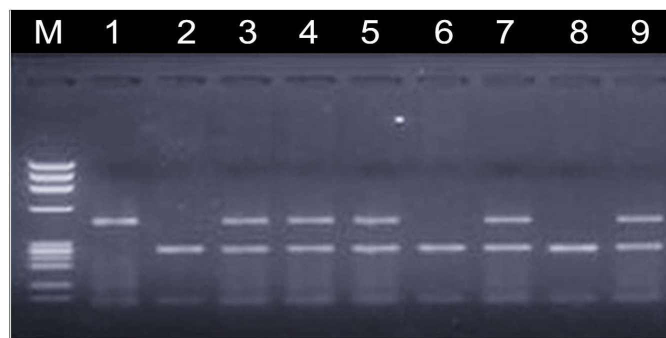

Figure 1: Characteristic banding pattern obtained for single and multiple drug resistant strains using multiplex PCR.

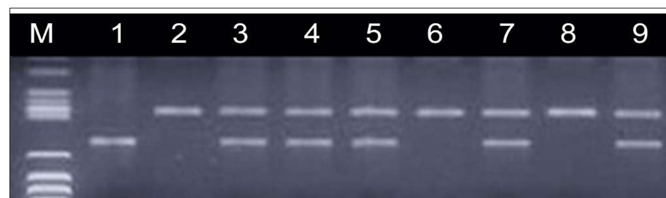

Figure 2: Characteristic banding pattern obtained for EMB resistant strain using multiplex PCR.

multiplex PCR taking MGIT culture system as gold standard are shown in Table III.

A characteristic ethidium bromide stained 1% agarose gel displaying banding pattern obtained after multiplex PCR containing primer pairs for RIF *(rpoB)*, INH *(katG)* and EMB *(embB)* gene. A single band of 305bp specifies resistance to RIF. A single band of 458bp specifies resistance to INH. A single band of 306bp specifies resistance to EMB. Two bands of 305bp and 458bp specify the resistance to RIF and INH. Two bands of 306bp and 458bp specify the resistance to EMB and INH, respectively. Lane M: 100bp DNA ladder, Lane 1: SDR (INH), Lane 2, 6 and 8: SDR (RIF), Lane 3-5, 7 and 9: MDR (RIF and INH).

A characteristic ethidium bromide stained 1% agarose gel displaying banding pattern obtained after multiplex PCR containing primer pairs for RIF *(rpoB)*, INH *(katG)* and EMB *(embB)* gene. A single band of 305bp specifies resistance to RIF. A single band of 458bp specifies resistance to INH. A single band of 306bp specifies resistance to EMB. Two bands of 305bp and 458bp specify the resistance to RIF and INH. Two bands of 306bp and 458bp specify the resistance to EMB and INH, respectively. Lane M: 100 bp DNA ladder, Lane 1: SDR (EMB).

DISCUSSION

In the present research, authors evaluated a multiplex PCR that allowed simultaneous detection of MTB resistance to RIF, INH and EMB in 84 MTB resistant isolates. Selected primers were for three loci at which RIF, INH and EMB resistance associated mutation for *rpo B* (531), *kat G* (316), and *emb B* gene (345) were most frequently observed.

Table III: Comparison of sensitivity, specificity, NPV, PPV and DA between phenotype and genotype-based characterisation of drug resistant MTB isolates.

Resistance pattern	Mutiple PCR	Culture		Sensitivity	Specificity (%)	PPV (%)	NPV (%)	DA (%)
		R	S					
MDR	R	9	0	50	100	100	33	59
	S	9	4					
RIF	R	17	0	100	100	100	0	100
	S	0	4					
INH	R	28	0	71	100	100	26	74
	S	11	4					
EMB	R	6	0	60	100	100	50	71
	S	4	4					

Sensitivity of multiplex PCR for detection of rifampicin resistance varies from region to region. In this study, sensitivity and specificity was found to be 100% comparable to similar study conducted in China with sensitivity of 81.5% and specificity of 92% for RIF.[19] One national study conducted in the Aga Khan Hospital, Karachi that compared phenotypic with genotypic molecular test (line probe assay PCR) with sensitivity 92.5% and specificity of 98.2% for RIF.[20]

It has been well established that most INH-resistant isolates possess mutation in *kat G* or *inh A* or both.[21] In this study, the most prevalent mutation at 458 bp region of kat G encompassing codon 315 was used. The total of 39 INH resistant isolates were identified in MGIT 960, 28 (42%) of which were found to contain mutation in *kat G*. In contrast, the remaining 11 (17%) had no mutation within 315, the target fragment of *kat G* gene. The sensitivity and specificity for *kat G* gene in this study was 71% and 100%, respectively. Similar study conducted in China with sensitivity and specificity of 41% and 100% for isoniazid resistant *kat G* gene.[17]

In this study, 306bp region of *emb B* gene for ethambutol was used. Out of 10 EMB resistant isolates identified in MGIT 960 system, 6 (10%) showed mutated bands in multiplex PCR for *emb B* gene. The sensitivity and specificity was 60% and 100%, respectively in this study. Very few reports have evaluated this *emb B* gene using MGIT system and multiplex PCR. However, this study evaluates emb B gene for ethambutol resistant as well.

In this study, simultaneous detection of MDR for *kat G* and *rpo B* was used. Out of the 18 MDR isolates identified in MGIT 960 system, 11 (14%) showed mutated bands in multiplex PCR, with sensitivity and specificity of 50% and 100% respectively in multiplex PCR. A similar study conducted for MDR showed sensitivity and specificity of 34% and 99%, respectively.[17]

In this study, out of total 84 isolates, 66 (79%) isolates had concordant results on both the methods (culture phenotypic method) or genotypic (multiplex PCR tests). Individually, INH had 38% while RIF had 17% concordance. The results of this study are similar to study by Ali *et al*. in which there was 84% concordance for INH and 100% for RIF.[17]

The main focus of this study was time-saving by multiplex PCR when compared with culture. Overall time-saving by multiplex PCR in our study was 8 to 13 days. This time saving is very important as it can help the clinicians in initiation of early therapy and thus controlling the spread of drug resistant tuberculosis (MDR or XDR) isolates.

This study has two main limitations. First, it was carried out on smear positive specimens only. Secondly, it was single-centre study and the representation of MDR isolates was small *i.e.* 21% of the total isolates.

CONCLUSION

Multiplex PCR is useful gadget for quick determination of drug resistant TB in specimens, hence permitting an initial therapeutic approach. However, for accurate management of patients, phenotypic method should be used to confirm results.

ETHICAL APPROVAL:

The study was ethically approved by Institutional Ethical Committee

CONFLICT OF INTEREST:

Authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:

SS, GZ: Did the project.
SY: Concept.
WH, UK: Peer viewed written stuff.
TK: Provided sources for research.

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