Molecular detection of mutations in isolates of multidrug resistant tuberculosis and tuberculosis suspects by multiplex allele specific PCR

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Abstract: For lowering prevalence of drug resistance it is necessary to diagnose TB in tuberculosis sputum suspect patients instead of TB-cultured samples which required a long time of culturing. Comparison of the results of drug resistant bacterial genes in both tuberculosis suspect sputum and multi-drug resistant DNA isolates detected by MAS-PCR. In the current study, the genetic mutations linked with INH, RIF as well as EMB drugs were detected by MAS-PCR simultaneously in MDR as well as TB suspect sputum isolates. 175/291 samples belonged to MDR and 116/291 samples belonged to tuberculosis suspect group. In all the isolates, presence of *Mycobacterium* tuberculosis-species (100%) was confirmed by targeting *hupB* gene. In MDR group, maximum prevalence of gene mutation was detected in *rpoB531* (92.57%) and *embB306* (97.71%) while in TB-suspect group, equal percentage (96.55%) of mutation was detected in *rpoB531* and *embB306* by MAS-PCR. Collectively, *rpoB531* (n=274, 94.15%) and *embB306* (n=283, 97.25%) mutation were observed in maximum tuberculosis cases. MAS-PCR technique yielded reliable results and showed massive Isoniazid, Rifampicin and Ethambutol drugs resistance in TB-isolates from Pakistan; hence it can be used in clinical laboratories with high burden of tuberculosis to detect drug resistance rapidly and cost effectively.

Keywords: Multidrug resistant tuberculosis, multiple allele specific PCR, *Mycobacterium tuberculosis*, mutations.

INTRODUCTION

Tuberculosis is a fatal, infectious disease due to which a large number of deaths have been occurred all over the world since ages ago (WHO, 2004). Every year, almost 1.8 million people perish because of TB, which depicts per day 4,500 casualties, a vast majority belongs to Pakistan, India, China, Bangladesh and Indonesia (Dye C, 2006). Tuberculosis is a burning health issue in Pakistan making it to stand at 4th position encompassed by 22 countries with high TB load (WHO, 2014). TB prevalence is approximately 350 per 100, 000 individuals in Pakistan (WHO, 2010). In year 2009 the number of individual died were 60, 000 in Pakistan (WHO, 2010). *Mycobacterium tuberculosis* is responsible for spread of pulmonary tuberculosis in humans (Vinay *et al*., 2007). *M. bovis* has zoonotic origin and may cause tuberculosis in humans like *M. tuberculosis* (Acha *et al*., 2003).

Although chest X-ray along with smear microscopy as well as LJ- media culturing are some of the widely used conventional methods for investigation of TB bacteria (Gupta *et al*., 2004) but more precise techniques are required which not only authenticate the *Mycobacterium* species but also define the drug resistance mechanism. Bacterial drug resistance is identified by a lengthy process of bacilli culturing which required six to eight weeks (Laszlo *et al*., 1997). Genetic mutations are possible to detect in short interval due to the advancement in molecular detection methods (Victor *et al., 2002*). TB becomes MDR-TB when the resistance against isoniazid and rifampicin drugs develops simultaneously (WHO, 2000). More number of MDR cases means difficulty in controlling the tuberculosis emergence rate because of less effectiveness of first line anti-TB drugs and it takes to more number of deaths due to failure of treatment ultimately taking to more disease transmission to the healthy persons (Schluger, 2000). Various molecular methods viz DNA sequencing (Kapur *et al.*, 1995) single strand polymorphism conformation (Scarpellini *et al.*, 1997) PCR-RFLP (El-Hajj *et al*., 2001) plus allele-specific PCR (Mokrousov *et al., 2003*) are used for mutation detections and each method has its own benefits as well as minor draw backs.

The most economical molecular method to detect a mutation in the gene is allele specific PCR as this method devoid of costly restriction enzymes as well as sequencings. A lot of parameters are causing the emergence of MDR-TB and some of the important ones are inappropriate diagnosis, drug dosing and time span of disease treatment (Jacobs, 1994). The tuberculosis bug after becoming resistant gets more chances of survival against the drug regimen (Chandrasekaran *et al.*, 1992) thus not only the cost of treatment increases but also the

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difficulty level of cure increases as well (Kailash *et al.*, 2000). Once the resistance is developed the treatment of disease faces issues like availability of anti-TB drugs, as the second line drugs are required in combination with first line drugs which makes the treatment not only more costly but also decreases the cure rate as compared to the treatment with first line drugs (Nathanson *et al*., 2006).

Curing the MDR-TB due to high fatality rate of 5-80% is not easy (Faustini *et al.*, 2006). When *rpoB* gene region RRDR mutates particularly at positions (516, 526 and 531) then it results into resistance against rifampicin, whereas resistance to isoniazid drug occurs due to mutation in *katG* (codon 315) and promoter-15 region (*mabA-inhA),* while resistance to ethambutol drug turns out due to mutation of *embB* gene at codon position 306 (Yang *et al.,* 2005).

In the current research, the identification and differentiation of *MTB* species from *M. bovis* was conducted by targeting a histone-like protein gene in PCR. MAS-PCR, the cost effective and economical technique which detects simultaneously INH, RIF and EMB resistance, was used in the current research. Genetic mutations linked with INH, RIF, and EMB were identified by targeting *katG*, *rpoB,* and *embB* genes respectively in multiple allele specific PCR.

MATERIALS AND METHODS

Ethics approval statement

Participants in the study were informed and given with the consent; moreover permission was taken from the institution where the study was conducted.

Sampling

Sputum specimens (n, 291) were assembled from patients coming from different localities of county to Ghulab Devi Chest Hospital, Lahore, Pakistan. The sampling and MAS-PCR testing was performed from 2013 to 2015.

DNA isolation and quantification

The total genomic DNA in all the samples was isolated by using column based (TIANamp Beijing, Cat No. DP304) genomic DNA isolation kit. Through NanoDrop method, quality as well as quantity of genomic DNA (1µL of purified DNA) was examined.

M. tuberculosis and M. bovis molecular detection

The differentiation of *M. tuberculosis* from *M. bovis* was performed by targeting *hupB* gene (Rv2986c) in PCR. MDR tuberculosis samples were tested in a previous study (Hameed *et al*. 2017 data to be published). The samples of suspect tuberculosis group were tested. The primers S 5'GTATCCGTGTGTCTTGACCTATTTG 3' and N 5'GGAGGGTTGGGATGAACAAAGCAG 3' produced PCR products 645bp and 618bp from *M. tuberculosis* species & *M. bovis* respectively as earlier reported by the (Prabhakar *et al*., 2004). S primer with M 5' GCAGCCAAGAAGGTAGCGAA 3' produced 318bp and 291bp fragments regarding *hupB* gene of *MTB* and *M. bovis* respectively.

PCR buffer (1X), 2.5mM ($MgCl_2$), 0.2 mM(dNTPs), primers M, N, S (100pmol each), 2.5U (taq polymerase), isolated DNA (0.1µg) and total volume was kept 50µL with deionized sterilized water. The PCR temperatures included at 94°C for 4 minutes initial denaturation with subsequent 40 cycles and each denaturation comprised of 30Seconds at temperature of 94°C, while annealing of one minute at 60°C, extension of 50 seconds and at temperature of 72°C. Finally at the end, 7 minutes polymerization at 72°C and at 4°C the reaction was terminated.

Multiple allele specific-PCR

MAS-PCR was conducted for all (n, 291) TB samples by using primers described earlier in table 1 by (Mokrousov *et al.,* 2002). Amplified products of genes in allele specific PCR comprised of 170 bp (*rpoB* gene, codon 531), 185 bp (*rpoB,* codon526), 218 bp (*rpoB,* codon 516); 292 bp (*katG,* codon 315); 270 bp (promoter -15 region); 335 bp (*embB,* codon 306) (table 3; fig. 3, 4). The PCR fragments of respective sizes were amplified in allele specific PCR when mutations were absent and vice versa.

MAS-PCR reaction mixture contained PCR buffer (1X), dNTPs (0.2 mM), $MgCl_2$ (2.5mM), primers (all ten primers) 10pmol each, *taq* polymerase enzyme (2.5U), isolated DNA (0.2µg) and total volume was kept 50µL with deionized sterilized water. The PCR temperatures included at 94°C for 4 minutes initial denaturation with subsequent 40 cycles and each comprised a 30 seconds denaturation at 94°C temperature, further one minute annealing at 65°C with an extension of 50 seconds where temperature was 72°C. The reaction was terminated with a 7 minutes final polymerization at 72°C and at 4°C. After MAS-PCR the samples were run on agarose gel (1.5%) while (UVtech, Inc. UK) gel documentation system was used for photographs.

RESULTS

Molecular differentiation of M. tuberculosis from M. bovis

The tuberculosis suspect group samples (n, 116) were tested in the current research and all were *M. tuberculosis* and no detection of *M. bovis* (table 2). (The 175 samples of MDR tuberculosis group were already tested and in all these MDR samples *M. tuberculosis* was detected (Hameed *et al*. 2017) data unpublished to date and not presented here and all the samples were *M. tuberculosis* and there was no detection of *M. bovis*). *M. tuberculosis* was differentiated from *M. bovis* by the amplification of

(*hupB*, Rv2986c) a histone-like protein gene in PCR. The primers S and N produced PCR products 645bp and 618bp respectively for *MTB* and *M. bovis* whereas primers S+M in combination produced 318bp and 291bp fragments for the differentiation of two species of *Mycobacteria* (table 2; fig. 1, 2).

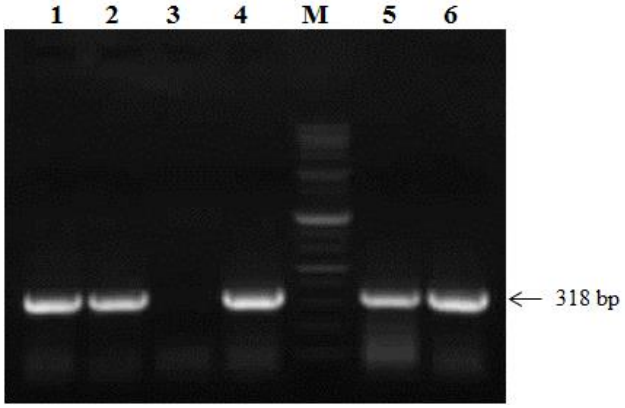

Fig. 1: Molecular detection of *Mycobacterium tuberculosis* by targeting *hupB* gene in PCR by primers M and S. Key: Lane1-2, 4-6: *M. tuberculosis* positive cases showing 318bp amplified band; Lane3: Negative control; M: DNA marker (Enzynomics, Cat No. DM003).

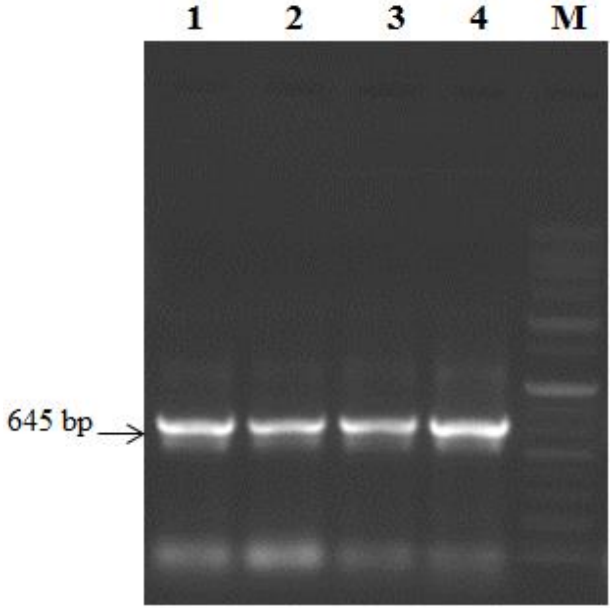

Fig. 2: Molecular detection of *Mycobacterium tuberculosis* by targeting *hupB* gene in PCR by primers N and S. Key: Lane1-4: *M. tuberculosis* positive cases showing 645 bp amplified fragment; M: DNA marker (Enzynomics, Cat No. DM003).

Multiple allele specific-PCR

Mutations in *rpoB* gene at three codon positions viz 531, 526, 516 caused resistance to drug rifampicin. The mutation in *rpoB* gene linked with rifampicin resistance at codon position 531 was found in maximum tuberculosis cases 274 (94.15%) while mutation at codon position 526 and 516 in *rpoB* gene were detected in 194 (66.66%) and 171 (58.76%) tuberculosis cases respectively (table 3) whereas 180 (61.85%) and 196 (67.35%) tuberculosis cases were found positive when mutation in *katG* gene at codon 315 and promoter regions occurred respectively (table 3). Mutation in *embB* gene at codon position 306 is related to resistance against ethambutol and detected in 283 (97.25%) tuberculosis cases (table 3). There was no pre-knowledge or record of resistance when MAS-PCR applied to tuberculosis suspect group (n, 116). The results indicated highest prevalence of *rpoB* gene mutation at codon 531 and *embB* gene at codon 306 (table 3) hence in these suspect cases multidrug resistance was detected and culturing test can be omitted for these cases.

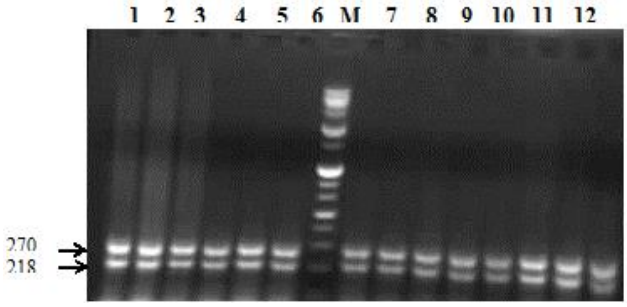

Fig. 3: Multiple allele specific PCR for showing mutations in genes *rpoB* (codons 526, 531), *katG* (codon 315) and *embB* (codon 306) of *Mycobacterium tuberculosis*. Key: Lane1-6: Tuberculosis patients samples of tuberculosis suspect group showing mutations in genes *rpoB* (codons 526, 531), *katG* (codon 315) and *embB* (codon 306) of *Mycobacterium tuberculosis* while genes *rpoB* (codon516) and *InhA* (mabA-*inhA*:-15) representing 218bp and 270bp fragments respectively exhibited no mutation; Lane7-12: MDR tuberculosis patients samples showing mutations in genes *rpoB* (codons 526, 531), *katG* (codon 315) and *embB* (codon 306) of *Mycobacterium tuberculosis* while genes *rpoB* (codon516) and *InhA* (*mabA-inhA*:-15) representing 218bp and 270bp fragments respectively exhibited no mutation; M: DNA marker (Enzynomics, Cat No. DM003).

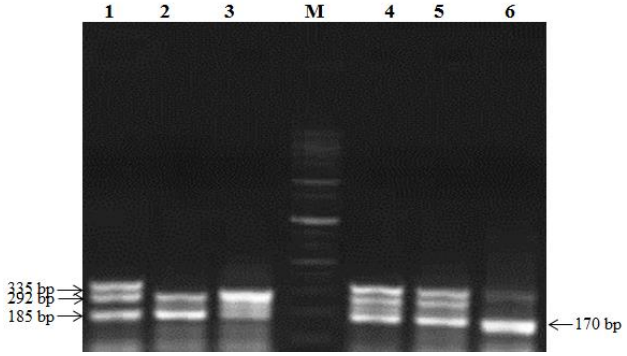

Fig. 4: Multiple allele specific PCR for showing mutations in different *Mycobacterium tuberculosis* genes. Key: Lane1-3: MDR tuberculosis patients samples; Lane4-6: Tuberculosis patients samples of suspect group; Lane1, 4, 5: Tuberculosis patients samples showing

Table 1: Primers used in multiple allele specific PCR.

Sr.#	Targeted Position	Drug	PCR Product (bp)	Forward Primer (5'-3')	Reverse Primers (5'-3')
1	*rpoB*516	Rifampicin	218	CAGCTGAGCCAATTCATGGA	TTGACCCGCGCGTACAC
2	*rpoB*526		185	CTGTCGGGGTTGACCCA	
3	*rpoB*531		170	CACAAGCGCCGACTGTC	
4	*KatG*315	Isonizid	292	GCAGATGGGGCTGATCTACG	ATACGACCTCGATGCCGC
5	*InhA (mabA-inhA*: -15)		270	CACCCCGACAACCTATCG	GCGCGGTCAGTTCCACA
6	*embB*306	Ethambutol	335	GGCTACATCCTGGGCATG	GAGCCGAGCGCGATGAT

Table 2: Molecular differentiation of *M. tuberculosis* and *M. bovis* by targeting of *hupB* gene in TB suspect group (n = 116).

Mycobacterium type	Primers combination used	PCR amplified fragment size (bp)	No. of positive cases	No. of negative cases	Prevalence (%)
M. tuberculosis	N+S	645	116	0	100
M. bovis		618	0	116	0.0
M. tuberculosis	M+S	318	116	0	100
M. bovis		291	0	116	0.0

*The primers used were earlier described by Prabhakar *et al*. (2004).

Table 3: Multiplex allele specific PCR results of MDR tuberculosis and suspect groups.

Gene and respective drug	Product size	Detection Target	No. of mutations for MDR group (n=175)	No. of mutations for TB Suspect group (n=116)	Collective (%) resistance n=291
rpoB (Rifampicin)	170bp	*rpoB*531	162 (92.57%)	112 (96.55 %)	274 (94.15%)
	185bp	*rpoB*526	102(58.28%)	92 (79.31 %)	194 (66.66%)
	218bp	*rpoB*516	125 (71.42%)	46 (39.65 %)	171 (58.76%)
katG (Isonizid)	292bp	*katG*315	120 (68.57%)	60 (51.72 %)	180 (61.85%)
inhA (Isonizid)	270bp	*mabA inhA*: -15	109 (62.28%)	87 (75 %)	196 (67.35%)
embB (Ethambutol)	335	*embB*306	171 (97.71%)	112 (96.55 %)	283 (97.25%)

mutations in gene *rpoB* (codons 516, 531) and *InhA* (mabA-inhA:-15); Lane2,3: Tuberculosis patients samples showing mutations in gene *rpoB* (codons 516, 531), *InhA* (*mabA-inhA*:-15) and *embB* (codon 306); Lane6: Tuberculosis patient sample showing mutations in gene *rpoB* (codons 516, 526), *InhA* (*mabA-inhA*:-15) and *KatG* (codon 315); M: DNA marker (Enzynomics, Cat No. DM003).

DISCUSSION

Pakistan ranks fourth in the world regarding tuberculosis prevalence and there is need to establish reliable molecular detection method of tuberculosis and resistance associated mutations in sputum samples as well as cultured samples. In the current research, the primers used for MAS-PCR performance were adopted as described by Yang *et al*., 2005 to detect rifampicin, isoniazid and ethambutol associated genetic mutations by targeting *rpoB, katG* along with promoter -15 region (*mabA-inhA*) and *embB* genes respectively. However, Yang *et al*. 2005 was unable to test these primers of MAS-PCR on genomic DNA isolated directly from tuberculosis patients and tested MAS-PCR on genomic DNA isolated after cultured samples only. In the current research, MDR-TB group sputum cultured samples (n=175) and tuberculosis suspect group with no history of drugs resistance (n= 116) direct sputum samples were subjected to genomic DNA isolation and MAS-PCR and in both cases results were found to equally acceptable and no nonspecific amplifications were observed in case of both types of samples i.e., sputum culture samples and sputum non cultured samples. In MDR group, *rpoB*531 (n=162, 92.57%) and *embB*306 (n=171, 97.71%) mutations were observed in maximum cases while in case of tuberculosis suspect group *rpoB*531 and *embB*306 (n=112, 96.55%) were found maximum as determined by multiple allele specific PCR. Collectively, *rpoB*531 (n=274, 94.15%) and *embB*306 (n=283, 97.25%) mutation were observed in maximum cases of tuberculosis cases. So, in MDR-TB and non MDR-TB i.e., tuberculosis suspect group, *rpoB*531 and *embB*306 gene mutations were predominated in patients. It is not necessary that the results which were presented in context of Pakistani population are same as in European or any other populations due to the fact that geographical distances,

risk factors and environment may change the spectrum of disease in different geographical locations.

In the current study, the patients included in both MDR and tuberculosis suspect group were earlier diagnosed by chest X-ray and AFB test but not passed through any confirmatory molecular test for the detection of *M. tuberculosis* as well as *M. bovis.* Therefore in the current study, all the TB samples of both groups were subjected to PCR based molecular test as narrated earlier by the Prabhakar *et al*., 2004 for the differentiation of *MTB* species from *M. bovis*.

The presence of *M. bovis* was negative in all TB samples of both the groups, possibly due to the reason that majority of patients have no animal contact and living in urban zones where animals were not kept in homes. Pakistan is included in high tuberculosis burden countries and molecular methods described in the current paper for detection of *MTB* species and *M. bovis* along identification of mutations in drugs resistance associated genes needed to be further validated on other populations where tuberculosis burden is high as in different geographical regions the pattern of mutations may vary with need of additional genomic targets in future studies.

The limitation for the current research was sampling; as the samples were collected from the hospital treating the patients coming from different localities of the country that's why a very high resistance rate was assumed so the results cannot be declared as a generalized results of whole community.

CONCLUSION

It is concluded from the current study that MAS-PCR has shown excellent results of drug resistance detected both in MDR-TB as well as suspect-TB isolates from Pakistan. As the technique required only the primers & PCR standards along with equipment of electrophoresis, so it can be used in the clinical laboratories of the world with high tuberculosis prevalence. The procedure of performance is easy, cost effective, and provides results in short time with more accuracy as compareD to other MDR-TB detection methods which are time consuming with less reliability of the results.

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