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Research Article

Amplification Refractory Mutation System – Polymerase Chain Reaction for Rapid Detection of *rpoB* Gene Mutations in *Mycobacterium tuberculosis*

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Abstract

Multidrug-resistant tuberculosis (MDR-TB) has become a serious worldwide threat including in Nepal. MDR-TB refers to the pathological condition whereby *Mycobacterium tuberculosis* becomes resistant to the first line of drug treatment i.e. rifampin and isoniazid. Resistance to rifampin (RIF) is mainly caused by the mutations in the *rpoB* gene which codes for the β-subunit of RNA polymerase. In this study, Amplification Refractory Mutation System – Polymerase Chain Reaction (ARMS – PCR) technique has been used to detect mutations in the *rpoB* gene of *Mycobacterium tuberculosis*. Total DNA samples of 34 phenotypic MDR-TB were subjected to ARMS – PCR using three different codon specific primers (516, 526 and 531). These three codons occupy large portion of total mutation responsible for rifampin resistance. Out of the total DNA samples, all were bearing mutation in at least one of the three codons mentioned. Of those bearing mutation, the highest number had mutation in codon 531 (97.05 %) followed by codon 516 (17.64 %) and finally in codon 526 (11.76%) respectively. Hence, ARMS – PCR may be used as an alternative diagnostic technique for detection of rifampin resistance in *Mycobacterium tuberculosis* strains, especially for a developing country like Nepal.

Keywords: ARMS-PCR; Multiple drug resistance; Mycobacterium tuberculosis; Rifampin; *rpoB*.

Introduction

Tuberculosis (TB) is regarded as a historical disease, which has a long and continuing record of causing worldwide morbidity and mortality especially in developing countries, including Nepal (Chadha, 2009). Infection by the bacillus *Mycobacterium tuberculosis* leads to the chronic disease with aerial mode of transfer between people (Zaman, 2010). The bacterium usually attacks lung called pulmonary tuberculosis but it can also attack other parts of the body such as the kidney, spine, and brain called extra pulmonary tuberculosis (Farer *et al.,* 1979). The nature of symptoms depends upon the site where the bacterium inhabits. In the cases of pulmonary TB, it may cause symptoms, such as chronic cough, pain in the chest, haemoptysis, weakness or fatigue, weight loss, fever, and night-sweats (Zaman, 2010).

When the *M. tuberculosis* organism develops resistance to at least Isoniazid and Rifampin, considered as the most effective medication against tuberculosis, they are referred to as Multidrug-resistant tuberculosis. The emergence of primary drug resistance in a patient who has never before been treated for tuberculosis would be due to infection by drug-resistant bacilli. On the contrary, secondary (acquired) drug resistance arises in a patient, who initially had drug-susceptible *M. tuberculosis*, through inappropriate or inadequate treatment, noncompliance to a prescribed regimen or poor absorbance of prescribed medication (Gangadharam, 1993).

Resistance to Rifampicin (RIF)

It is mainly caused by the mutations in the *rpoB* gene coding the β subunit of RNA polymerase**.** Rifampicin is the most important drug available for TB treatment. It inhibits gene transcription by binding to the β subunit of the DNA dependent RNA polymerase, encoded by the *rpoB* gene. Most *rpoB* mutations occur through point mutations within a 81 bp rifampicin resistance- determining region (RRDR), located between codons 507 and 533 with the most common changes being observed in codons Ser531Leu, His526Tyr and Asp516Val (O'Sullivan *et al.,* 2005; Sekiguchi *et al.,* 2007). Furthermore, more than 90% of RIF-resistant

isolates are also resistant to isoniazid; therefore, rifampin resistance can be assumed to be a surrogate marker for MDR TB, which identifies MDR strains (Drobniewski and Wilson, 1998).

Amplification Refractory Mutation System – Polymerase Chain Reaction (ARMS – PCR)

The amplification-refractory mutation system (ARMS) is a simple, rapid and reliable method for detecting any mutation involving single base changes or small deletions (Ferrie *et al*., 1992). ARMS – PCR is based on the use of sequence-specific PCR primers that allow amplification of test DNA only when the targ*et al*lele is contained within the sample. Following an ARMS reaction the presence or absence of a PCR product is diagnostic for the presence or absence of the targ*et al*lele (Newton *et al.*, 1989).

The working principle behind ARMS- PCR is that oligonucleotides which are complementary to a wild type DNA sequence will only function as primers in PCR under optimized conditions, however, the mutated DNA sequence won't get amplified (Fan *et al*., 2003). The protocol has been followed for the detection of several genetic polymorphisms including 1-antitrypsin deficiency (Newton *et al*., 1989), CFTR gene mutation (Ferrie *et al*., 1992), apolipoprotein E genotypes (Donohoe *et al*., 1999), and K-ras mutation (Carpenter *et al*., 1996). With respect to its limitation, ARMS PCR can be used for the detection of the mutation only but not its nature, hence cannot substitute for the results obtained through DNA sequencing (Fan *et al*., 2003).

Present scenario of TB in Nepal

In Nepal, the incidence of all forms of TB was estimated to be 136/100,000 population, while the incidence of new smear-positive cases was at 57/100,000 in 2013/2014. The proportion of new cases with multidrug-resistant TB (MDR-TB) was 2.2% among new cases and 15.4% among retreatment cases based on survey carried out in 2011/12 in Nepal. In 2014, total of 349 MDR – TB patients were confirmed. Nepal is a landlocked country in Southeast Asia, bounded to the north by China and to the south by India, sharing an open border with India. India and China had the largest numbers of TB cases (23%, and 10% of the global total, respectively). Every year, a large number of people of Nepal and India cross the border for various purposes, such as work, study, trade, pilgrimage, cultural visits, and so on. Since drug resistance rates on one side of the border can impact the other side of the border, a high proportion of MDR-TB in Nepal may reflect the possible dissemination of infection from surrounding two countries, mainly from India (NTP Annual Report, 2014; WHO Report, 2015).

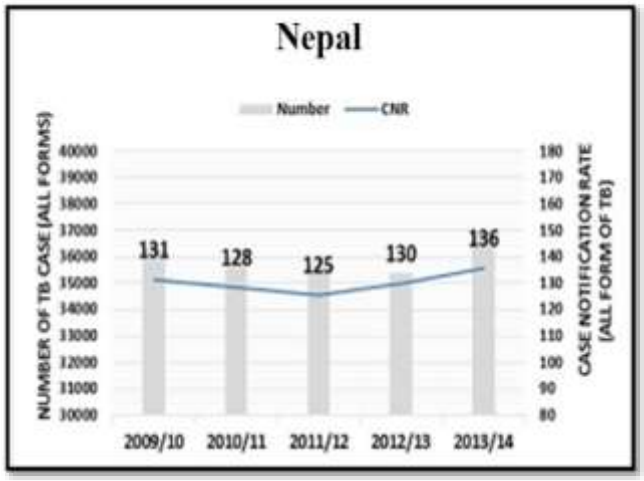

Fig. 1: Time-series of national TB notification numbers and rates in Nepal, 2009/10 -2013/14 [Adapted from NTP Annual Report, 2014]

Material and Methods

Due to the infectious nature of the pathogen, rather than live cultures, a total of 34 cell lysates were obtained from Global Hospital, Lalitpur, Nepal. DNA was purified from the cell lysates and used for PCR amplification.

ARMS – PCR for Detection of rpoB Mutation

Purified DNA from the Mycobacterial samples were subjected to PCR using ARMS primer. Upon completion of PCR, the amplicon were then run on 2 % Agarose gel supplemented with 0.5 µg/ml of Ethidium bromide, alongside 100 base pair ladder (Cat. N3231S) and visualized under ultra-violet (UV) light on a transilluminator. The condition for PCR reaction has been provided in Table 1.

Table 1: PCR condition for detection of mutation in *rpoB* gene by ARMS PCR

Stage	Steps	Temperature	Time	No. of cycles
1.	Initial denaturation	95°C	5 min	1
2.	Denaturation	94°C	35 sec	35
	Annealing	56°C	35 sec	
	Extension	72°C	35 sec	
3.	Final Extension	72°C	10 min	1
4.	Hold	4°C	∞	-

Table 2: PCR primer for ARMS PCR

Gene	Primer	Sequence
rpoB	Control Forward Primer	5′– CGAATATCTGGTCCGCTTGC – 3′
	Common Reverse Primer	5′– GTCGACCACCTTGCGGTACG – 3′
	ARMS – 516 primer	5′– CAGCTGAGCCAATTCA**C**GGA – 3′
	ARMS – 526 primer	5′– CGCTGTCGGGGTTG**T**CCC – 3′
	ARMS – 531 primer	5′– ACCCACAAGCGCCGAC**A**GTC – 3′

Primer Design

The primers used in this study were designed as described by Fan *et al.*(2003).The rationale of primer designing for ARMS PCR is that a single nucleotide mismatch at the 3'-OH extremity of the annealed forward primer renders Taq DNA polymerase unable to extend the primer in the PCR under appropriate conditions. Thus, the absence of the specific PCR product, with a positive result for the internal control, reveals a deviation from the wild-type DNA sequence. An additional deliberate mismatch (shown in Table 2 as bold and underlined base) adjacent to the 3′-OH terminus of the ARMS primer was introduced in order to enhance discrimination between normal and mutant alleles (Fan *et al.*, 2003).

Results and Discussion

Detection of *rpoB* mutation by ARMS PCR

For detection of mutation in *rpoB* gene, the ARMS primers employed was complementary to the corresponding sequence of the wild-type gene except for one additional deliberate mismatch at the fourth nucleotide from the 3′-OH terminus of the primer. This, in turn would create two mismatched nucleotides at the 3′ end between the ARMS primer and the mutated codon. A single mismatch at the fourth nucleotide from the 3’ end of the ARMS primer would have little influence on the yield of PCR products, whereas the mismatch at the 3′-OH extremity of the primer is obstinate to extension by the Taq DNA polymerase so that amplification from the mutant allele does not occur (Fan *et al.*, 2003).

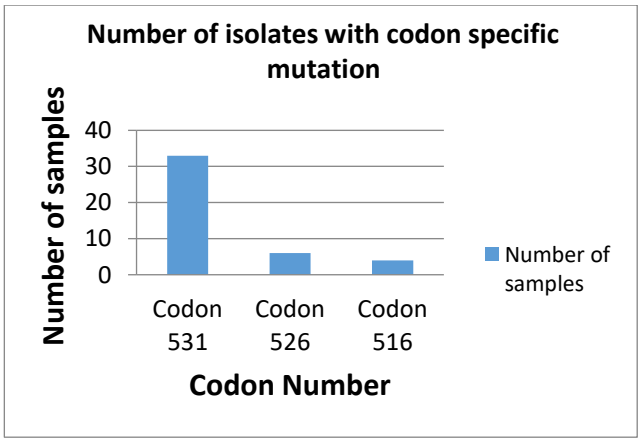

Fig. 2: Graph representing the number of phenotypic MTB samples with mutations in specific codons.

A total of 34 DNA samples of phenotypic MDR *Mycobacterium tuberculosis* were subjected to ARMS PCR assay. Each isolate had mutation in at least one of the codons namely 516, 526 or 531, with several samples showing cross mutations. Quantitatively, 33 samples had mutation at codon 531(97.05 % of total confirmed samples), 6 samples were found to bear mutation at codon 516 (17.64 % of total mutated samples) and the codon 526 was found to have been mutated in the least number of samples, being detected only among 4 samples (11.76% of total mutated samples) (Fig. 2). Hence, among the samples used in this study, the maximum number of mutation was detected to be in codon 531 while the least mutation was observed in the codon 526. Among 34 isolates considered in our study, the magnitude for frequency of mutation in the codons were found in following order 531>516>526.

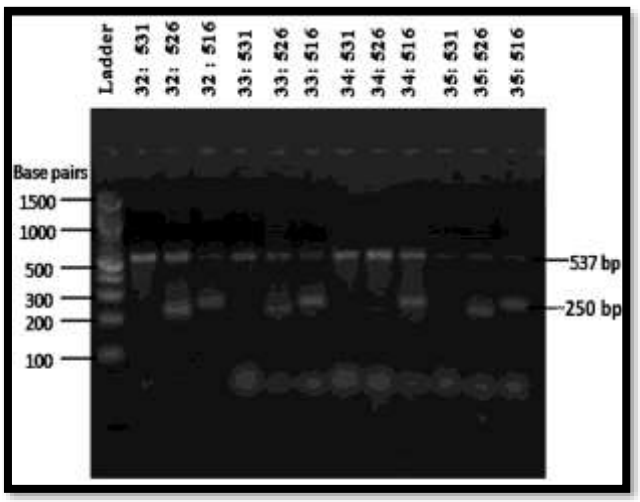

Fig. 3: Agarose Gel Electrophoresis (2 %) for amplicons obtained after PCR with control forward, common reverse and ARMS primers (531, 526 and 516). 100 base pair Ladder (Cat. No. N3231S). The code 32: 531 signifies that the PCR amplification for sample number 32 has been performed with 531 codon specific ARMS primer, control forward primer and common reverse primer.

ARMS PCR products of some strains with typical mutations in the codons being studied from the *rpoB* gene are shown in Fig. 3. Control forward primer and common reverse primer amplified 537 bp of *rpoB* gene containing RRDR region which contained three codons i.e. codon 516, 526 and 531. Three codon specific primers were used in each sample separately for detection of mutation in respective codons. Control forward primer and common reverse primer were used in each of the samples, along with codon specific

primer, for amplification of 537 bp region of *rpoB* gene serving as an internal control to avoid false negative results. Double bands were observed in the samples with non - mutated codons corresponding to a band of approximately 261 bp and 537 bp for the codon specific primer of 516, approximately 230 bp and 537 bp for the codon specific primer belonging to 526 codon, and the band length of 261 bp and 537 bp was visible for the codon 531. Single band representative of the amplification by control forward and common reverse corresponding to a band length of 537 bp was observed in case of samples with mutated codon.

Summary of the analyzed data showing the number of isolates with codon specific as well as multi codon mutation as gathered from the ARMS PCR assay has been presented in the Table 3.

Table 3 Table showing the isolates with mutation in the specific codons as detected by the ARMS PCR.

Samples	Mutation		
	Codon531	Codon526	Codon516
02r	√	-	-
03r	√	-	-
04r	√	-	-
05r	√	-	-
06r	√	-	-
07r	√	-	-
08r	√	-	√
10r	√	√	-
11r	√	√	-
12r	√	-	-
13r	√	-	-
14r	√	-	-
16r	√	√	-
17r	√	-	-
18r	√	-	-
21r	√	-	√
24r	√	-	√
26r	√	-	√
29r	√	-	-
31r	√	-	-
32r	√	-	-
33r	√	-	-
34r	√	√	-
35r	√	-	-
36r	√	-	-
37r	√	-	-
38r	√	-	-
40r	-	-	√
42r	√	-	-
43r	√	-	-
44r	√	-	-
45r	√	-	-
46r	√	-	-
49r	√	-	√

Conclusion

The present study was conducted for molecular analysis of the *rpoB* gene responsible for generation of rifampicin resistant *Mycobacterium tuberculosis*. The results showed that ARMS PCR for detection of mutation in *rpoB* may be used as an alternative to sequencing under resource constrained environments. However, detection of *rpoB* gene mutation alone cannot be the surmise for confirmation of multiple drug resistance in the given organism. Furthermore, only a certain portion of *rpoB* universally regarded to be mutation prone region was analyzed, i.e. the mutations in other parts were left undetected. Hence further studies are required for analysis of mutations in the wholesome gene sequence in larger data sample sets

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